

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371ATTORNEY'S DOCKET NUMBER
01142.0101U.S. APPLICATION NO.
(If known, see 37 CFR 1.5)

09/786056

INTERNATIONAL APPLICATION NO.

PCT/US99/20011

INTERNATIONAL FILING DATE

September 1, 1999

PRIORITY DATE CLAIMED

September 1, 1998

TITLE OF INVENTION

ENHANCED FUNCTIONAL EXPRESSION OF G PROTEIN-COUPLED
RECEPTORS

APPLICANT(S) FOR DO/EO/US

Mark Henry PAUSCH; Margaret LAI; Sanford SILVERMAN; Camelia
BIRSAN; William BAUMBAUCH; Eugene TSENG; Eileen Marie
KAJKOWSKI and Bradley Alton OZENBERGER

Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A sequence listing containing 14 sheets and statement Accompanying Sequence Listing (1 page)
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).
20. ☒ Other items or information:
 - a. ☒ Copy of cover page of International Publication No. WO00/12704 A3.
 - b. ☐ Copy of Notification of Missing Requirements.
 - c. ☒ **Preliminary Amendment to Follow** - Total claim and Independent Claim count will change, as a result of the amendment.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.
PCT/US99/20011ATTORNEY'S DOCKET NUMBER
01142.010121. ☒ The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO\$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO\$860.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO\$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
but all claims did not satisfy provisions of PCT Article 33(1)-(4)\$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
and all claims satisfied provisions of PCT Article 33 (1)-(4)\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$860.00

Surcharge of **\$130.00** for furnishing the oath or declaration later than
months from the earliest claimed priority date (37 CFR 1.492 (e)).☐ 20 ☐ 30

\$

CLAIMS

NUMBER FILED

NUMBER EXTRA

RATE

Total Claims

63

- 20 =

x \$18.00

\$

Independent Claims

7

- 3 =

x \$80.00

\$

MULTIPLE DEPENDENT CLAIM(S) (if applicable)

+ \$270.00

\$270.00

TOTAL OF THE ABOVE CALCULATIONS =

\$1130.00

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.

\$

SUBTOTAL =

\$1130.00

Processing fee of **\$130.00** for furnishing the English translation later than
months from the earliest priority date (37 CFR 1.492(f)).☐ 20 ☐ 30

\$

TOTAL NATIONAL FEE =

\$1130.00

Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by
an appropriate cover sheet (37 CFR 3.28, 3.31). **\$40.00** per property.

\$

TOTAL FEES ENCLOSED =

\$1130.00

Amount to be refunded: \$


charged: \$

- a. ☒ A check in the amount of \$ 1130.00 to cover the above basic fee and multiple claims fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to
Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.
1300 I Street, N.W.
Washington, D.C. 20005-3315


SIGNATURE
Ernest F. Chapman, Reg. No. 25,961
NAME/REGISTRATION NO.

DATED: March 1, 2001

18 NOV 2001

PATENT
Attorney Docket No. 01142.0101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
)
Mark H. PAUSCH et al.)
)
Application No.: 09/786,056) Group Art Unit: Not Yet Assigned
)
Filed: March 1, 2001) Examiner: Not Yet Assigned
)
For: ENHANCED FUNCTIONAL EXPRESSION)
OF G PROTEIN-COUPLED RECEPTORS)

Assistant Commissioner for Patents
Washington, D.C. 20231

**RESPONSE TO NOTIFICATION TO COMPLY WITH REQUIREMENTS FOR
PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCES AND/OR
AMINO ACID SEQUENCE DISCLOSURES**

Sir:

On July 12, 2001, the Office mailed a Notification to Comply with Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures. The Notification requires Applicants to submit an initial or substitute computer readable form (CRF) of the Sequence Listing.

Enclosed is a computer readable form (CRF) of the Sequence Listing, and a copy of the Notice to Comply. The undersigned verifies that the computer readable form of the Sequence Listing and the paper copy of the Sequence Listing submitted on March 1, 2001, are the same.

If there are any fees due in connection with the filing of this Response that are not enclosed with the Response to Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US), which is being filed at the same time as this Response, please charge the fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: 

Matthew T. Latimer

Reg. No. 44,024

(202) 408-4495

matthew.latimer@finnegan.com

Date: November 13, 2001

Enclosures:

Computer Readable Form of Sequence Listing
Copy of Notification

LAW OFFICES

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WASHINGTON, DC 20005
202-408-4000



JC17 Rec'd PCT/PTO 20 JUN 2001

PCT

PATENT
Attorney Docket No. 1142.0101-00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
)
Mark H. PAUSCH et al.)
)
Application No.: 09/786,056) Group Art Unit: Not Yet Assigned
)
Filed: March 1, 2001) Examiner: Not Yet Assigned
)
For: ENHANCED FUNCTIONAL EXPRESSION)
OF G PROTEIN-COUPLED RECEPTORS)

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examination of this application, please amend it as follows:

IN THE CLAIMS:

Please cancel claims 2, 5, 14, and 28 without prejudice to, or disclaimer of, the subject matter contained therein.

Please amend claims 1, 3, 6-8, 13, 17, 26, 27, and 42-45 as follows:

1. (Amended) A yeast host cell comprising a constitutively active heterologous G protein-coupled receptor.

3. (Amended) The host cell according to claim 1, wherein the heterologous G protein-coupled receptor is modified at an intracellular domain of the G protein-coupled receptor.

6. (Amended) The host cell according to any one of claims 1, 3, or 4, wherein the heterologous G protein-coupled receptor is an orphan receptor.

7. (Amended) The host cell according to claim 1, wherein the heterologous G protein-coupled receptor is modified at amino acid residues Asp-Arg-Tyr in the domain proximal to the second intracellular loop of the G protein-coupled receptor.

8. (Amended) The host cell according to claim 3, wherein the modified G protein-coupled receptor is a human alpha 2A adrenergic receptor and the modification comprises a point mutation of threonine to lysine at amino acid residue 373.

13. (Amended) A method for screening compounds capable of binding to G protein-coupled receptors, said method comprising (a) subjecting the host cell according to claim 1 to a test compound; and (b) measuring the effect of the test compound on cell growth.

17. (Amended) The host cell according to claim 15, wherein the mutation results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G

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protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery, or proper plasma membrane localization.

26. (Amended) A method for screening compounds capable of binding to G protein-coupled receptors, said method comprising (a) subjecting the host cell according to claims 15, 18, 20, or 21 to a test compound; and (b) measuring the effect of the test compound on cell growth.

27. (Amended) A method for expressing constitutively active heterologous G protein-coupled receptors in a yeast host cell comprising:

- (a) transforming the host cell with a vector comprising a DNA sequence encoding a modified heterologous G protein-coupled receptor, wherein the modification results in a constitutively active G protein-coupled receptor; and
- (b) culturing the transformed host cell to permit expression of the heterologous G protein-coupled receptor.

42. (Amended) An isolated DNA molecule encoding a chimeric G alpha protein, wherein the DNA molecule comprises a first nucleic acid sequence encoding the amino terminal domain of a G alpha protein of a first species, and a second nucleic acid sequence encoding the carboxy terminus of a G alpha protein of a second species, which is different from the first species.

43. (Amended) The DNA molecule according to claim 42, wherein the amino terminal domain of the G alpha protein of the first species comprises an interaction domain for a G beta protein, for a G gamma protein, and for an effector molecule.

44. (Amended) An isolated DNA molecule encoding a chimeric G alpha protein, wherein a first nucleic acid sequence encoding the five carboxy terminal amino acids of a G alpha protein from a first species is substituted for a second nucleic acid sequence encoding the five carboxy terminal amino acid sequences of a G alpha protein from a second species, which is different from the first species.

45. (Amended) A polypeptide encoded by the DNA molecule of claim 42 or 44.

REMARKS

By this Preliminary Amendment, claims 2, 5, 14, and 28 are canceled, and claims 1, 3, 6-8, 13, 17, 26, 27, and 42-45 are amended. Support for the amendments to claims 1 and 27 comes from the specification, as originally filed, and claims 5 and 28, for example. Claims 3 and 6-8 are amended to maintain proper dependencies. Claims 13, 17, 26, and 42-45 are amended to correct typographical errors and improve clarity. No new matter is added by the amendments. Currently, claims 1, 3, 4, 6-13, 15-27, and 29-51 are pending in this application.

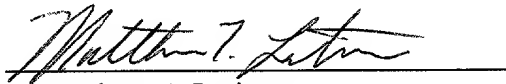
Applicants respectfully request prompt and favorable examination of this application. If the Examiner believes anything further is necessary in order to place this application in better condition for prompt examination, Applicants request that their undersigned representative be contacted at the telephone number or e-mail address listed below.

If there is any fee due in connection with the filing of this Preliminary Amendment, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By:



Matthew T. Latimer

Reg. No. 44,024

(202) 408-4495

matthew.latimer@finnegan.com

Date: June 20, 2001

Attachment:
Appendix

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APPENDIX

~ 09/786,056

1. (Amended) A yeast host cell comprising a constitutively active heterologous G protein-coupled receptor.
3. (Amended) The host cell according to claim [2] 1, wherein the heterologous G protein-coupled receptor is modified at an intracellular domain of the G protein-coupled receptor.
6. (Amended) The host cell according to any one of claims 1, 3, or 4 [to 5], wherein the heterologous G protein-coupled receptor is an orphan receptor.
7. (Amended) The host cell according to claim [5] 1, wherein the heterologous G protein-coupled receptor is modified at amino acid residues Asp-Arg-Tyr in the domain proximal to the second intracellular loop of the G protein-coupled receptor.
8. (Amended) The host cell according to claim [5] 3, wherein the modified G protein-coupled receptor is a human alpha 2A adrenergic receptor and the modification comprises a point mutation of threonine to lysine at amino acid residue 373.
13. (Amended) A method for screening compounds capable of binding to G protein-coupled receptors, said method comprising [the steps of] (a) subjecting the host cell according to claim 1 to a test compound; and (b) measuring the effect of the test compound on cell growth.
17. (Amended) The host cell according to claim 15, wherein the mutation results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the receptor to interact with cell [desensitization] desensitization or sequestration-internalization machinery, or proper plasma membrane localization.

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WASHINGTON, DC 20005
202-408-4000

26. (Amended) A method for screening compounds capable of binding to G protein-coupled receptors, said method comprising [the steps of] (a) subjecting the host cell according to claims 15, 18, 20, or 21 to a test compound; and (b) measuring the effect of the test compound on cell growth.

27. (Amended) A method for expressing constitutively active heterologous G protein-coupled receptors in a yeast host cell comprising:

- (a) transforming the host cell with a vector comprising a DNA sequence encoding a modified heterologous G protein-coupled receptor, wherein the modification results in a constitutively active G protein-coupled receptor; and
- (b) culturing the transformed host cell to permit expression of the heterologous G protein-coupled receptor.

42. (Amended) An isolated DNA [sequence] molecule encoding a chimeric G alpha protein, wherein the DNA [sequence] molecule comprises a first nucleic acid sequence encoding the amino terminal domain of a G alpha protein of a first species, and a second nucleic acid sequence encoding the carboxy terminus of a G alpha protein of a second species, which is different from the first species.

43. (Amended) The DNA [sequence] molecule according to claim 42, wherein the amino terminal domain of the G alpha protein of the first species comprises an interaction domain for a G beta protein, for a G gamma protein, and for an effector molecule.

44. (Amended) An isolated DNA [sequence] molecule encoding a chimeric G alpha protein, wherein a first nucleic acid sequence encoding the five carboxy terminal amino acids of a G alpha protein from a first species is substituted for a second nucleic acid sequence encoding the five carboxy terminal amino acid sequences of a G alpha protein from a second species, which is different from the first species.

45. A polypeptide encoded by the DNA molecule of claim 42 or 44.

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09/786056

IN THE EUROPEAN PATENT OFFICE
BEFORE THE INTERNATIONAL SEARCHING AUTHORITY

JC02 Rec'd PCT/PTO 01 MAR 2001

Atty. Docket No: 01142.0101-00304

In re International Application: PAUSCH, MARK H. et al.

International Application No.: PCT/US99/20011

International Filing Date: September 1, 1999

For: ENHANCED FUNCTIONAL EXPRESSION OF G PROTEIN-COUPLED RECEPTORS

European Patent Office
Storage and Retrieval of Amino
Acid and Nucleotide Data
Room POH09
Patentlaan 2
P.B. 5818
NL-2280 HV Rijswijk
The Netherlands

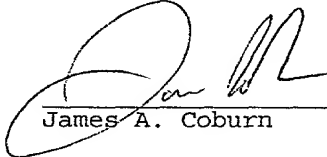
STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing.

Respectfully submitted,

Dec. 17, 1999
Date


James A. Coburn

HARBOR CONSULTING
Intellectual Property Services
1500A Lafayette Road
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(800) 318-3021

ENHANCED FUNCTIONAL EXPRESSION OF
G PROTEIN-COUPLED RECEPTORS

STATEMENT OF RELATED APPLICATIONS

5 This application hereby claims the benefit of United States provisional application S.N. 60/098,704 filed September 1, 1998. The entire disclosure of this provisional application is relied upon and incorporated by reference herein.

FIELD OF THE INVENTION

10 This invention relates to mutant G protein-coupled receptors with constitutively activating mutations that permit detection of the receptors' functional activity in the absence of activating ligands, host cells that contain mutations that promote the functional activity of the G protein-coupled receptors, host cells expressing such receptors, vectors useful for making such cells and methods of making and using the same. This invention also relates to modified G alpha proteins, and particularly to chimeric yeast-mammalian G alpha proteins, host cells expressing these modified G alpha proteins, vectors for making such cells and methods of making and using the same.

BACKGROUND OF THE INVENTION

20 The actions of many extracellular signals, such as neurotransmitters, hormones, odorants, and light, are mediated by a triad of proteins which has been identified in organisms from yeast to mammals. This triad consists of a receptor, coupled to a trimeric guanine nucleotide-binding regulatory protein (G protein), which in turn is coupled to a cellular effector. These receptors have seven transmembrane domains and are named for their association with the G protein as "G protein-coupled receptors" ("GPCRs").

25 The regulatory G proteins are comprised of three subunits: a guanylnucleotide binding α subunit; a β subunit; and a γ subunit. *B.R. Conklin and H.R. Bourne (1993)*. G proteins cycle between two forms, depending on whether GDP or GTP is bound to the α subunit. When GDP is bound, the G protein exists as a heterotrimer, 30 the $G\alpha\beta\gamma$ complex. When GTP is bound, the α subunit dissociates, leaving a $G\beta\gamma$

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complex. Importantly, when a $G\alpha\beta\gamma$ complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of disassociation of the bound $G\alpha$ subunit from the $G\beta\gamma$ complex increases. The free $G\alpha$ subunit and $G\beta\gamma$ complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways. Examples of these downstream cellular effector proteins include, among others, adenylate cyclases, ion channels, and phospholipases. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. *H.G. Dohlman et al. (1991).*

Because of their ubiquitous nature in important biochemical pathways, the G protein-coupled receptors represent important targets for new therapeutic drugs. In turn, the discovery of such drugs will necessarily require screening assays of high specificity and throughput, termed high-throughput screening (HTS) assays. Screening assays utilizing microorganisms, such as yeast strains, genetically modified to accommodate functional expression of the G protein-coupled receptors offer significant advantages in research involving ligand binding to numerous receptors implicated in various disease states.

These screening assays depend on functional expression of the G protein-coupled receptors in the desired host cell. Functional expression of wild type G protein-coupled receptors was developed in a *Saccharomyces cerevisiae* cell system based on the observation that yeast utilizes G protein-coupled receptors and heterotrimeric G proteins to regulate the mating process. *M. Pausch (1997).* Haploid yeast cells detect the presence of peptide mating pheromones secreted by potential mating partners through binding to G protein-coupled pheromone receptors encoded by the *STE2* and *STE3* genes. Activated pheromone receptors catalyze dissociation of a heterotrimeric G protein, encoded by the *GPA1* (α), *STE4* (β), and *STE18* (γ) genes. Gpa1p-mediated negative regulation is thereby relieved, allowing the complex of Ste4p and Ste18p to activate a signal transduction pathway composed elements of a mitogen activated protein kinase (MAP kinase) pathway. *M.C. Gustin et al. (1998).* The pathway is composed of MAP kinase homologs encoded by the *FUS3* and *KSSI* genes, and upstream regulatory protein kinases encoded by the *STE7*, (MAP kinase

- 3 -

kinase or MEK) and *STE11* (MEK kinase) genes. The result of activation of this pathway is cell cycle arrest and transcriptional induction of pheromone-responsive genes. Mutations in elements of the MAP kinase pathway abolish these responses.

The product of the *FAR1* gene mediates cell cycle arrest in response to pheromone. *FAR1* encodes a negative regulator of G1 cyclins and is thought to serve as the primary interface between the pheromone response pathway and cell cycle regulatory machinery. *M.A. Peter et al. (1993)*. Deletion of the *FAR1* gene allows for continued cell growth and transcriptional induction of pheromone-responsive genes in the presence of an activated mating signal transduction pathway.

The yeast cell response to chronic stimulation of the pheromone response pathway is induction of specific desensitization or adaptation mechanisms. The primary adaptation mechanism is mediated by the product of the *SST2* gene, *H.G. Dohlman et al. (1997)*, the prototypical RGS protein. Yeast cells lacking functional Sst2p exhibit hypersensitivity to the presence of pheromone and inability to recover from pheromone-induced cell cycle arrest.

Detecting the yeast cells that respond to agonist requires an additional modification, the addition of pheromone responsive reporter genes. In order to confer a selectable phenotype, the protein-coding segment of the *HIS3* gene, which encodes an enzyme required for histidine biosynthesis, was used to replace the *FUS1* protein-coding region. Thus, expression of this enzyme necessary for growth on medium lacking histidine is made dependent on activation of the pheromone response pathway. Agonist activation of this *FUS1-HIS3* reporter gene in *his3* cells would be expected to produce a growth response on medium lacking in histidine. In the absence of agonist activation, the *FUS1-HIS3* reporter gene is largely inactive, little His3 gene product is produced, the cells make insufficient histidine and therefore cannot grow. In order to permit screening of samples that contain histidine, e.g. natural products extracts, a reporter gene that confers inducible G418 resistance may be included. Other reporter genes functional in this system include *E. coli LacZ*, which encodes beta-galactosidase, and green fluorescence protein. Direct screening for antagonists may be accomplished using the stably integrated *FUS2-CAN1* reporter gene.

- 4 -

5 This technology was previously employed to characterize somatostatin SST2, adenosine A2a, cholecystokinin CCK-B and growth hormone releasing hormone receptors expressed in yeast. *L.A. Price et al. (1995), L.A. Price et al. (1996), M.H. Pausch et al. (1998), E. M. Kajkowski et al. (1997)*. Through its use, novel SSSTR2-selective agonists and antagonists were developed from SAR based on peptide analogs of somatostatin, *R.T. Bass et al. (1996)*, and via screening of a d-amino acid peptide library. *W.R. Baumbach et al. (1998)*. Modified versions of the technology have been employed to characterize ligands for LPA, melatonin and chemokine receptors. *J.R. Erickson et al. (1998), T. Kokkola et al. (1998), C. Klein et al. (1998), T. J. Baranski et al. (1999)*.

10 However, microorganisms transformed with wild-type receptors may perform poorly in growth assays, exhibiting, for example, the inability to interact with the heterotrimeric G protein, inappropriate localization and/or desensitization. Many GPCRs are phosphorylated in response to chronic and persistent agonist stimulation which often leads to desensitization followed by sequestration or internalization of the receptors. Desensitization of GPCRs causes uncoupling from interaction with heterotrimeric G proteins. This process is mediated by a variety of regulatory receptor protein kinases, including G protein-coupled receptor kinases (GRK), protein kinase A (PKA), protein kinase C (PKC), and casein kinases (CK). Internalization involves removal of GPCRs from the plasma membrane via receptor-mediated endocytosis. Internalized receptors may be recycled back to the cell surface, or delivered to a lysosomal/vacuolar compartment for degradation. The ubiquitin-mediated degradative pathway is also involved in this process. The ultimate result of receptor phosphorylation and sequestration/internalization is often cell growth arrest, which significantly reduces the utility of the genetically modified microorganism in screening assays.

25 In addition to poor performance in growth assays, other problems hamper the utility of many wild type G protein-coupled receptors screening assays. Recent efforts to clone GPCRs from humans and other species have revealed the presence of a large number of DNA sequences that appear to encode GPCRs for which no cognate ligand has been identified. These so-called orphan receptors are a challenging problem for

- 5 -

the drug discovery industry. In the usual manner of developing drug screening assays, a GPCR with a known cognate agonist is expressed in a heterologous expression system, e.g., mammalian cells, yeast, etc. The ability of the GPCR to functionally interact with the intracellular heterotrimeric G proteins is determined via activation of the GPCR with its cognate agonist and subsequent measurement of resulting G protein-mediated activation of effector enzyme activity. Successful activation of effector activity validates the screening assay, permitting the drug discovery scientist to have confidence that the GPCR is appropriately expressed and functional. The difficulty with the orphan GPCR lies in the fact that, since no cognate agonist is known for the orphan GPCR, its expression and functional activity cannot be verified.

SUMMARY OF THE INVENTION

It is an object of this invention to provide modified G protein-coupled receptors and G proteins that function well in high throughput screening assays, implemented in any eukaryotic cell, preferably yeast cells. Thus, a first aspect of the present invention is directed to nucleotide sequences encoding a G protein-coupled receptor which has been modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery, and/or to appropriately localize to the plasma membrane. In preferred embodiments, such modifications lead to improved agonist-stimulated growth-promoting ability. One specific modification of the nucleotide sequence encoding a G protein-coupled receptor encompassed by this invention is a mutation in any intracellular domain or membrane region proximal to internal domains.

It is another object of this invention to provide modified orphan G protein-coupled receptors that can function in high throughput screening assays even though its specific cognate ligand is unknown. Thus, in a second aspect of this invention, the nucleotide sequence encoding a GPCR encompassed by this invention encodes a modification of the amino acids involved in receptor activation resulting in production of a constitutively active GPCR. Such mutations may occur throughout the GPCR sequence. Particularly interesting are mutations in regions proximal to and within the

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second and third intracellular loops and within the transmembrane domains. One specific modification is a mutation in a conserved motif found in rhodopsin family GPCRs that is involved in GPCR activation and known as the DRY box, i.e., the domain proximal to the second intracellular loop encoding amino acid residues Asp-Arg-Tyr.

This invention is also directed to chimeric GPCRs in which intracellular domains of heterologous GPCRs that provide favorable G protein coupling properties or domains not subject to the yeast cell desensitization and/or sequestration/internalization machinery are used to replace comparable domains in GPCRs of interest. This invention also relates to expression vectors comprising the modified nucleotide sequences and to host cells transformed therewith.

In a third aspect of this invention, host cells, such as yeast cells, may be mutated via deletion or point mutation to promote improved coupling of heterologous GPCRs to heterotrimeric G proteins and/or eliminate receptor kinases or other components of the desensitization and/or sequestration/internalization machinery. One such modification of the host cells according to this invention involves a mutation that affects the ratio or nature of sterols in the membrane. Other examples of modifications to host cells according to this invention include (1) mutations in host cell genes encoding the heterotrimeric G protein that improve coupling with heterologous GPCRs; (2) mutation of genes encoding regulatory receptor protein kinases, including G protein-coupled receptor kinases, protein kinase A, protein kinase C and/or casein kinases, that cause a reduction in receptor phosphorylation; and (3) mutations in genes encoding components of the endocytic and/or degradative pathways including ubiquitin mediated pathways that reduce receptor sequestration/internalization and/or degradation. The result of these mutations is a host cell that exhibits improved capacity to support functional expression of heterologous GPCRs.

It is a fourth aspect of this invention to provide host cells that express chimeric G proteins that function well in HTS assays. These host cells exhibit improved coupling of G proteins to heterologous GPCRs expressed therein and, thus, are useful to screen for coupling selectivity of expressed heterologous GPCRs, or to demonstrate

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the functional expression of orphan GPCRs in the absence of agonist. One preferred chimeric G protein is composed of sequences from Gpa1, the yeast G alpha protein, in which the five carboxy-terminal amino acids have been replaced with those from various heterologous G protein alpha subunits, including those from mammals and other vertebrate species, insects and other invertebrate species, and various fungal species. Other examples include chimeric G protein alpha subunits composed of an amino-terminal G protein beta and gamma subunit binding domain from Gpa1 fused to carboxy-terminal receptor binding domains from heterologous G alpha proteins.

Additional aspects of this invention include methods of functionally expressing an orphan GPCR in a host cell with activation by agonist. Constitutively active GPCRs can promote growth in the absence of agonist, thus, demonstrating that they are functionally expressed. Functional expression may be further enhanced by expressing constitutively active GPCRs in host cells that contain a chimeric G alpha protein, thereby improving GPCR-G alpha protein coupling selectivity. In combination, positive results from both types of experiments provide convincing evidence that a GPCR may be functionally expressed in a host cell. With this evidence in hand, HTS assays using the wild type GPCR may be pursued with confidence that the GPCR is functionally expressed and capable of responding to the presence of ligands. Therefore, host cells containing expressed constitutive GPCRs may be used in an improved method of assaying compounds to determine the effects of ligand binding. Compounds that promote growth over that attributable to the action of the constitutive GPCR may be agonists, those that inhibit growth may be antagonists or inverse agonists.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A depicts the results of liquid culture assays on yeast cells containing the rat M3 muscarinic acetylcholine receptor (MAR) using MAR agonist carbachol (CCh). Yeast cells containing a M3 MAR with a deletion in the third intracellular loop (IC3Δ) produced an agonist-dependent growth response, while the wild type MAR did not, indicating that the M3 MAR IC3Δ is functional GPCR.

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FIGURE 1B depicts the results of liquid culture fluorescence induction assays on yeast cells containing the rat M3 MAR IC3Δ and the FUS2-GFP reporter plasmid using the MAR agonist carbachol (CCh). A dose-dependent increase in the expression of the green fluorescent protein is observed in response to CCh activation of the M3 MAR IC3Δ expressed in yeast.

FIGURE 2 depicts the results of liquid culture assays on yeast cells containing the *Drosophila* muscarinic acetylcholine receptor using the MAR agonist carbachol (CCh). Yeast cells containing a mutated *Drosophila* MAR containing the M3 MAR IC3Δ produced an agonist-dependent growth response while the wild type *Drosophila* MAR lacked an agonist-dependent yeast cell growth response.

FIGURE 3 depicts the results of an agar-based plate bioassay. **FIGURE 3A** shows a robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor. **FIGURE 3B** shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of a portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

FIGURE 4 depicts yeast cells transformed with rSSTR3 and with rSSTR3ΔIC3. **FIGURE 4A** demonstrates that yeast cells containing p426GPD-rSSTR3 show a weak response to somatostatin (S-14). **FIGURE 4B** demonstrates a much stronger response by yeast cells containing p426GPD-rSSTR3ΔIC3 assayed under similar conditions.

FIGURE 5 depicts the results of liquid culture assays on yeast cells containing wild type IC3Δ and a constitutively active mutant (CAM) IC3Δ human alpha2A adrenergic receptor using the alpha adrenergic receptor full agonist UK14304. Yeast cells containing the wild type and CAM IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that the IC3 deletion is functional.

FIGURE 6A depicts the results of liquid culture assays on yeast cells containing wild type IC3Δ and a constitutively active mutant (CAM) IC3Δ human alpha2A adrenergic receptor using the alpha adrenergic receptor full agonist UK14304 in the presence of AT (3-aminotriazole). **FIGURE 6B** depicts the results of liquid culture assays on yeast cells containing wild type IC3Δ and a constitutively active

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mutant (CAM) IC3Δ human alpha2A adrenergic receptor using the alpha adrenergic receptor partial agonist clonidine in the presence of AT. Only the CAM alpha2A-AR responds to clonidine, consistent with the increased responsiveness to agonist expected of a CAM GPCR.

FIGURE 7 depicts the results of an agar-based plate bioassay on yeast cells containing modified (CAM) M3-MAR compared to unmodified M3-MAR in the presence of MAR agonist, carbachol, and inverse agonist, atropine. **FIGURE 7A** shows that the CAM M3-MAR promotes an elevated yeast growth rate in the absence of agonist and a reversal of agonist-independent growth in the presence of inverse agonist, atropine. **FIGURE 7B** shows that atropine had no effect on the basal growth of the CAM M3-MAR in the presence of histidine.

FIGURE 8 depicts the results of liquid culture assays on yeast cells containing wildtype and carboxy terminally truncated rat NT1-neurotensin receptors using the neurotensin receptor agonist AcNT8-13. Truncation of the rat NT1-neurotensin receptor produces an agonist-dependent growth response that is more sensitive than that observed with the wildtype receptor.

FIGURE 9 depicts yeast cells containing the vasopressin receptor plasmids, embedded in agar in the absence (left) and presence (right) of 1 mM aminotriazole. **FIGURE 9A** depicts strain MPY578fc (ERG6) transformed with V2 receptor. **FIGURE 9B** depicts strain YML103 (*erg6*) transformed with V2 receptor. **FIGURE 9C** depicts strain MPY578fc (ERG6) transformed with V1a receptor. **FIGURE 9D** depicts strain YML103 (*erg6*) transformed with V1a receptor.

FIGURE 10 depicts yeast cells containing receptor plasmids, embedded in agar in the absence (left) and presence (right) of 1 mM aminotriazole. **FIGURE 10A** depicts strain MPY578fc (ERG6) transformed with melanocortin MC4 receptor. **FIGURE 10B** depicts strain YML103 (*erg6*) transformed with melanocortin MC4 receptor. These results demonstrate that the changes in membrane properties caused by the *erg6* mutation results in improved responses by vasopressin V2, and V1a receptors, and melanocortin MC4 receptors.

FIGURE 11 depicts the results of experiments in which heterologous GPCRs have been assayed in yeast strains bearing the chimeric G alpha proteins. For each

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indicated GPCR, + indicates that a dose-dependent growth response to cognate agonist was observed when assayed in the liquid format; - indicates no detectable agonist dose-dependent growth response. These results demonstrate the functional activity of chimeric G proteins coupled to heterologous GPCRs expressed in yeast.

FIGURE 12 depicts the agonist-independent growth response to high and low level adenosine A2a receptor expression in yeast cells containing various chimeric G alpha proteins. When expressed at high levels, adenosine A2a receptors promote growth of only a subset of yeast cells containing chimeric G proteins, indicating that this receptor retains G alpha protein coupling selectivity when expressed in yeast cells. The growth promoting activity is greatest when the adenosine A2a receptor is expressed at high levels and less when adenosine A2a receptor expression is reduced.

DETAILED DESCRIPTION OF THE INVENTION

Modified G Protein-Coupled Receptors

Nucleotide sequences encoding G protein-coupled receptors may be modified to improve the function of the GPCR by causing the receptor to couple more efficiently with the heterotrimeric G protein and/or to fail to interact with the cell desensitization and/or sequestration/internalization machinery, leading to activation of downstream signal transduction pathways. In higher eukaryotic cells, these pathways may include the stimulation or inhibition of adenylyl cyclase leading to changes in intracellular cyclic AMP concentrations, stimulation of phospholipase C β , leading to increases in intracellular inositol trisphosphate and/or calcium ion concentrations as well as changes in the activities of G protein regulated potassium and calcium channels, sodium/hydrogen exchangers and other membrane localized signaling proteins. In a preferred embodiment, such modifications lead to improved agonist-stimulated yeast cell growth-promoting ability. The improvement of GPCR-G protein coupling and/or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell provides a means to improve the function of wildtype heterologous GPCRs that fail to stimulate a useful yeast cell growth response. Thus, GPCRs that fail to function in their wild type form may be made to work by the methods of this invention.

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5 The improvement of GPCR-G protein coupling and or elimination of receptor phosphorylation and/or sequestration/internalization in the host cell may be assessed by using routine techniques, such as those described in the Examples set forth below or known to those of skill in the art. For example, improvement of the function of a mutated GPCR over wildtype may be quantified as a statistically significant increase in the signal-to-noise ratio and/or in the sensitivity of the liquid bioassay. The signal-to-noise ratio is determined by comparing the agonist-induced growth rate to the growth rate observed in the absence of agonist. A statistically-significant increase in the signal-to-noise ratio resulting from agonist-stimulation of a mutated GPCR over similar values obtained from cells containing a wildtype receptor indicates that the function of the mutated GPCR has been improved.

10 The sensitivity of the liquid bioassay is defined as the agonist concentration necessary to produce a half-maximal growth rate (ED50 or EC50). The sensitivity of the bioassay is increased if a mutated GPCR produces a half-maximal growth rate at an agonist concentration that is statistically significantly less than that required by the wildtype GPCR.

15 Similarly, the more qualitative agar based bioassay will reflect increases in signal-to-noise ratio and/or sensitivity due to agonist stimulation of mutated GPCRs. In the agar based bioassay, signal-to-noise ratio increases are determined by comparing the extent of growth within the agonist induced growth zone resulting from stimulation of mutated and wildtype receptor. The sensitivity of the bioassay is proportional to the radius of the growth zone. Since applied compounds diffuse radially from the site of application to the agar, agonist concentration varies with the square of the radius of the growth zone. Thus, a larger zone of growth in response to agonist activation of mutated GPCRs reflects an increase in sensitivity.

20 Any G protein-coupled receptor may be employed in practicing this invention. Examples of such receptors include, but are not limited to, adenosine receptors, somatostatin receptors, dopamine receptors, cholecystokinin receptors, muscarinic cholinergic receptors, α -adrenergic receptors, β -adrenergic receptors, opiate receptors, 25 cannabinoid receptors, histamine receptors, growth hormone releasing factor, glucagon, serotonin receptors, vasopressin receptors, melanocortin receptors, and

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neurotensin receptors. In certain preferred embodiments, the receptor is a muscarinic acetylcholine receptor and more preferably, the muscarinic acetylcholine receptor is of the M3 subtype.

Similarly, any suitable host cell may be transformed with the modified G protein-coupled receptors of this invention. Examples of suitable host cells are yeast cells, mammalian cells, insect cells, and bacterial cells. Preferably, the host cells are yeast cells, mammalian cells, or insect cells.

One generalizable method for improving the function of a GPCR expressed in a host cell is by modification or elimination of portions of the intracellular domains of the GPCR, such as the third intracellular loop (IC3) sequences of the G protein-coupled receptor. Because the desensitization and internalization machinery acts upon the intracellular domains of the GPCR, elimination of the intracellular domains of the GPCRs produces a more stable receptor expression. This has been demonstrated in experiments conducted in mammalian cells. Muscarinic acetylcholine receptors, including the M3 subtype, lacking a domain of their third intracellular loop thought to be involved in receptor internalization, are maintained in the plasma membrane to a greater extent than their wildtype counterparts. *Moro et al. (1993)*.

In addition to providing modified known GPCRs that function more efficiently (e.g., possess improved agonist-stimulated growth-promoting ability), this invention also provides modified orphan GPCRs whose functional activity can be verified even though its cognate ligand is unknown. Investigation into the mechanism of GPCR activation has lead to the proposal of a ternary model which accounts for the cooperative interactions between GPCR, G proteins and ligands. *Gether and B.K. Kobilka (1998)*. In this proposal, the unliganded GPCR may find itself in a unique state, R, that can undergo transition to at least two other equilibrium states: R*, an active state which is stabilized by agonists, and R°, an inactive state which is stabilized by inverse agonists. The active state is the form that is capable of stimulating heterotrimeric G proteins and subsequent effector enzyme activity. GPCRs in the R state find themselves in equilibrium between other states and therefore may undergo spontaneous transition to either R* or R° states in the absence of ligand.

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Experimental analysis of GPCR activation has led to the discovery of constitutively active GPCR mutants (CAMs). The CAM GPCRs have elevated intrinsic activity compared to wild type receptors consistent with a shift in the R to R^* equilibrium towards R^* , resulting in a CAM GPCR that is far more active in the absence of agonist than wild type. This permits the CAM GPCRs to interact with and activate intracellular heterotrimeric G proteins in an agonist-independent manner. Effector enzymes activated downstream can be used as an indirect measure of the intrinsic activity of the GPCR. In this way, the CAM GPCRs may be shown to be functional without resort to activation using cognate agonists. Due to the equilibrium nature of the R^* state of the CAM GPCRs, inverse agonists are capable of reversing the activation and stabilizing the R^o state, effectively inactivating the CAM GPCR. Neutral antagonists, drugs that block the binding of agonists and inverse agonists without affecting the level of constitutive activity of GPCR, have no effect on the activation state of the receptor.

Several distinct domains of GPCRs play a role in the activation process. *U. Gether and B.K. Kobilka (1998)*. Of particular interest are the domains proximal to and within the second and third intracellular loops of the GPCR. Proximal to the second intracellular loop and located at the cytoplasmic side of transmembrane domain 3 (TMD3) is the DRY motif, which is highly conserved in the rhodopsin GPCR family. Protonation of the acidic aspartate residue is thought to result in receptor activation. Indeed, mutation of this residue in the alpha 1a adrenergic receptor causes the receptor to become constitutively active. *A. Scheer and S. Cotecchia (1997)*. The greatest degree of constitutive activation is correlated with the hydrophobicity of the amino acid that replaces the Asp residue. Thus, replacement of the Asp residue with hydrophobic Ile, Leu, Met, or Val residues by site-directed mutagenesis produces a CAM alpha 1a adrenergic receptor.

The third intracellular loop also plays a role in GPCR activation. *Lefkowitz et al. (1993)*. Mutations in the C-terminal portion of this domain have the effect of producing CAMs. Site-directed mutations in both the alpha 1a adrenergic receptor and alpha 2a adrenergic receptor in the third intracellular loop have the effect of producing constitutive activity, perhaps due to release of constraints on activation that are

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maintained in this domain. Accordingly, this invention encompasses GPCRs that have been modified to produce constitutive activity. Preferred modifications are made to the DRY box or the third intracellular loop of the GPCR and result in a constitutively active receptor.

5 The membrane spanning helices also play a crucial role in ligand binding and activation of GPCRs. The role of these domains in receptor activation is indicated by the presence of constitutively activating mutations in the transmembrane helices. *P. Pauwels and T. Wurch (1998)*. Indeed, mutations that constitutively activate receptors have been found in 6 of 7 transmembrane helices.

10 Within the third transmembrane helix, a critical aspartic acid residue is known to be involved in binding of biogenic amine ligands and some peptides to their cognate GPCRs. A salt bridge between this aspartic acid residue and a lysine residue in transmembrane helix 7 restrains the activity of the $\alpha 1$ -adrenergic receptor. *Porter et al. (1998)*. Mutations that interfere in the formation of this salt bridge produce constitutive activation. Interestingly, triethylamine will act like a partial agonist, interfering in the formation of the restraining salt bridge. Such a mechanism may be useful for activating any GPCR that possesses a critical aspartic acid residue in its third transmembrane domain.

15 Additional amino acid residues within the third transmembrane domain may be mutated to cause constitutive activity. Using a yeast-based genetic screen, mutations in two conserved amino acids in the third transmembrane helix of the C5a receptor cause constitutive activity. *Baranski et al. (1999)*. The mutations exchanged the hydrophobic amino acids residues for those with hydrophilic character (I124 N, L127Q). Since hydrophobic character is conserved in similar positions in most
20 GPCRs, modification of these residues to those with hydrophilic properties may be a generalized method for activating GPCRs.

25 Mutations in the sixth transmembrane domain have also been demonstrated to cause constitutive activity. In the m5 muscarinic acetylcholine receptor, systematic mutagenesis of the sixth transmembrane domain has revealed a hot spot for
30 constitutive mutations. *Spalding et al., (1998)*. A cluster of amino acid residues that line one face of the sixth transmembrane domain appear to be involved in the

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formation of a ligand-dependent switch involved in GPCR activation. Identification of this hot spot suggests that mutagenesis of other receptors in the sixth transmembrane domain may lead to generalized method for activating GPCRs. In addition, mutations in receptors that affect ubiquitin-mediated or ubiquitin-independent degradation would be desirable for increasing signaling. More specifically, mutations analogous to those in *STE2* or *STE3* which alter PEST degradation signals would be most useful.

Modified Host Cells

This invention also encompasses modifications to a host cell by mutation of host cell genes or manipulation of the host cell environment in such a way as to allow G protein-coupled receptors to be expressed more efficiently. Examples of suitable host cells include yeast cells, mammalian cells, insect cells, and bacterial cells.

Since the pioneering work of *M.S. Brown and J.L. Goldstein (1976)*, it has been thought that the characteristics of membranes can affect endocytosis, i.e., internalization, of a variety of receptors. Mutations such as those described by *M.S. Brown and J.L. Goldstein (1976)* and *K. Tomita et al. (1985)*, or treatments such as those described by *A. Grider et al. (1996)*, that affect the ratio or nature of sterols in the membrane can alter endocytosis efficiency. Sterol depletion can also affect virus entry into the cells. *M.T. Marquardt and M. Kielian (1996)*. Cholesterol in mammalian membranes can modulate the function of G-protein coupled receptors by at least two mechanisms: changes in membrane fluidity and/or specific receptor-sterol interactions. *G. Gimpl et al. (1997)*. More specifically, it is known that receptor-mediated endocytosis is a major mechanism for recycling of G protein-coupled receptors, leading to desensitization to the continued presence of ligand. In the yeast *Saccharomyces cerevisiae*, mutations in the *erg6* gene, which alter the nature of membrane sterols, suppress defects in endoplasmic reticulum-golgi sorting. *K.G. Hardwick and H.R.B. Pelham (1994)*. Finally, yeast sterol mutants are reported to be defective in endocytosis and mating efficiency. *H. Riezman et al. (1997)*; *R. Gaber et al. (1989)*.

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Changes in the ratio or nature of sterols in host cell membranes may be affected in several ways and are within the scope of the invention. As noted above, one such mechanism to affect this change in sterol ratio is by mutation of host cell genes. Alternatively, changes in the environment may also alter the nature of ratio of sterol in the plasma membrane to permit the G protein-coupled receptors to be expressed more efficiently and are encompassed within the ambit of this invention.

In addition to altering cell sterols by the *erg6* deletion method, other examples of suitable host cell modifications include, but are not limited to: (1) using other sterol yeast mutants, such as *erg2*, *erg3*, *erg4*, and *erg5*, resulting in cells with different sterol alterations that can affect GPCR signaling; and (2) using a mutant of yeast, allowing the cells to grow in the presence of exogenously added sterols. Specifically, alterations in the *SUT1*, *PDX3*, *UPC1*, or (*UPC2* (*UPC20*)) genes will provide useful mutants that allow the host cells to grow in the presence of exogenously added sterols. In addition, any heme (*hem*) mutant can be used, particularly *hem1* and *hem3* mutants will allow the host cells to grow in the presence of exogenously added sterols. Suitable exogenously added sterols include, but are not limited to, cholesterol, and are added to the medium in which the cells are growing. The cells incorporate such sterols into their membranes and in the same manner as described in the examples below, heterologously expressed GPCR signal transduction is assayed in these cells.

In addition to modification of host cell sterols, other examples of modifications to host cells according to this invention include mutations in host cell genes encoding the heterotrimeric G protein that improve coupling with heterologous GPCRs; mutation of genes encoding regulatory receptor protein kinases, including G protein-coupled receptor kinases, protein kinase A, protein kinase C and/or casein kinases, that cause a reduction in receptor phosphorylation; and mutations in genes encoding components of the endocytic and/or degradative pathways including ubiquitin mediated pathways that reduce receptor sequestration/internalization and/or degradation. More specifically, conditional, or gain-of-function, or loss-of-function mutations in many genes can lead to increased signaling through the pheromone pathway. Such mutations affect endocytosis either directly or indirectly by affecting sorting of endosomal vesicles that may contain recycling receptors. The genes that

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would be most useful in this regard include, but are not limited to: *ASG7*, *DNM1*, *STE5*, *CDC30*, *DPM1*, *GAS1*, *SSO1*, *SSO2*, *SEC9*, *SRO7*, *RHO3*, *GCS1*, *GLO3*, *GLL4*, *AKR1*, *YCK1*, *YCK2*, *END3*, *UDH1*, *SCD5*, *END4*, *END5*, *VPS23*(=*STP22*), *SNF7*, *SNF8*, *VPS2*, *VPS28*, *VPS1*, *VPS8*, *VPS27*, *VPS36* and *KAP104*.

Chimeric G Alpha Proteins

This invention also encompasses chimeric G alpha proteins that improve the coupling efficiency between G proteins and G protein-coupled receptors and, therefore, can be used to enhance the functional expression of G protein-coupled receptors in a host cell. In a preferred embodiment, the chimeric G alpha protein is a yeast-mammalian G alpha protein encoded by a modified *GPA1* allele.

The types of G proteins to which a GPCR couples determines the effector output in response to agonist activation. Present methods for determination of the G protein coupling specificity of GPCRs are laborious and time-consuming since they require construction of multiple mammalian cell lines and assays of several effector protein activities. The present invention simplifies and accelerates the process of determining GPCR/G protein coupling specificity by using host cells that express a single chimeric G alpha protein. These host cells can be constructed by integrative replacement of a chromosomal locus, such as the *GPA1* locus in yeast, which encodes a G alpha protein. The object of the integrative replacement is to create a chimeric gene that encodes a G alpha protein in which the endogenous amino-terminal G $\beta\gamma$ and effector molecule interaction domain is fused to a carboxy terminal GPCR interaction domain encoded by DNA sequences from a mammalian G alpha subunit gene.

In yeast, the chimeric genes are preferably integrated into the *GPA1* locus. Steady-state *GPA1* mRNA and protein levels are determined by the degree of activation of the mating signal transduction pathway. *G.F. Sprague et al. (1992)*, *C. Dietzel et al. (1997)*, *I. Miyajima et al. (1987)*, *M. Nakafuku et al. (1987)*. In the absence of pheromone activation, the *GPA1* allele maintains a low basal level of expression. Upon activation of the pathway, *GPA1* mRNA and protein levels increase several fold leading to an immediate increase in sensitivity of the response. Further

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increases in Gpa1 levels in excess of that necessary to interact with the G $\beta\gamma$ subunits of the heterotrimeric complex, lead to down-regulation of the pathway. Thus, appropriate regulation of Gpa1 levels during the course of the mating response is important to determining the sensitivity and duration of the response.

5 The chimeric *GPA1* genes according to the present invention may contain a sequence that codes for the amino terminal G $\beta\gamma$ and effector molecule domain of *GPA1*, which is fused to another sequence encoding the carboxy-termini from a mammalian G protein alpha subunit, including but not limited to G α i2, G α i3, G α o, G α s, G α q, G α z, G α 11, G α 12, G α 13, G α 14, G α 15, and G α 16. Alternatively, the sequence encoding the 5 carboxy-terminal amino acids may be replaced with an equivalent coding sequence from a mammalian G protein alpha subunit, including but not limited to G α i2, G α i3, G α o, G α s, G α q, G α 11, G α z, G α 12, G α 13, G α 14, G α 15, and G α 16.

10 Chimeric G alpha proteins can be used to analyze the G protein coupling specificity of different GPCRs, including but not limited to the rat somatostatin SSTR2, rat adenosine A2a, rat muscarinic acetylcholine M2 and M3, *D. melanogaster* muscarinic acetylcholine M1, rat neurotensin NT-1, human vasopressin V2, rat cholecystikinin CCK-A and CCK-B, human gonadotropin releasing hormone GnRH, human melanocortin MCR4, human adrenergic α 2A, *Aplysia californica* octopamine OA1, human bombesin receptor related sequence 3 (BRS3), human histamine H3, and human β 2-adrenergic receptors. For example, yeast cells containing the chimeric *GPA1* genes discussed above can be transformed with plasmids conferring expression of a heterologous GPCR. The activities of cognate GPCR agonists can then be detected through dose-dependent induction of a pheromone-responsive *HIS3* reporter gene. Thus, host cells that express a chimeric G alpha protein provide a generally useful means of characterizing the G protein-coupling selectivity of G protein coupled receptors.

20 Representative embodiments of the invention are described in more detail in the following examples.

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Example 1. Functional Expression of A Mutated Rat M3 Muscarinic Acetylcholine Receptor (MAR) in Yeast

The third intracellular loops of GPCRs are thought to interact with and participate in the activation of G proteins upon agonist binding. *J. Wess (1997)*. Mutations in IC3 of the yeast mating pheromone receptors, Ste2 and Ste3 have profound effects on coupling the G proteins. *C. Boone et al. (1993)* and *C. Clark et al. (1994)*. Importantly, deletion of a portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in mammalian cells with retention of full ability to couple to the heterotrimeric G protein, Gq ($G\alpha\beta\gamma$). The mutated M3 MAR retains all external loops. Transmembrane domains (TMDs) and internal domains other than the IC3 are unchanged. The IC3, found between 5th and 6th membrane spanning helices, was the only domain modified. The bulk of this domain, 96 amino acids in the center of the IC3 (Ala273-Lys469), were deleted, leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, the third intracellular loop of the MAR containing the IC3 deletion (IC3 Δ) is 44 amino acids in length, compared to 240 amino acids in the IC3 of wild type M3 MAR. The improvement in functional expression may due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

In order to test the possibility that this IC3 Δ mutation would also improve functional expression in yeast, the DNA sequences encoding the wild type and IC3 Δ rat M3 MARs were cloned into proximity to the glycerol-phosphate dehydrogenase promoter in the yeast expression plasmid, p426GPD, by standard methods. Rat M3 MAR sequences were amplified by PCR using oligonucleotides containing 5' *Bgl*II (AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *Xho*I (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. The resulting PCR product was digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. To form the rat M3 IC3 Δ , three M3 MAR fragments were amplified by PCR. An amino-terminal coding fragment was amplified using oligonucleotides containing 5' *Bgl*II

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(AAAAGATCT AAA ATG TAC CCC TAC GAC GTC CCC) (SEQ ID NO: 1) and 3' *AgeI* (ATAGTCATGATGGTG ACCGGT ATGTAAGGCAGCGATC) (SEQ ID NO: 3) sites. A carboxy-terminal coding fragment was amplified using oligonucleotides containing 5' *PmlI* (GCCTTCATCAT CACGTG GACCCCTACACC) (SEQ ID NO: 4) and 3' *XhoI* (AAA CTCGAG CTA CAA GGC CTG CTC CGG CAC TCG C) (SEQ ID NO: 2) sites. An IC3 coding fragment was amplified using oligonucleotides containing 5' *AgeI* (CGATCGCTGCCTTTTACTT ACCGGT CACCATCATGACTAT) (SEQ ID NO: 5) and 3' *PmlI* (GTTGTAGGGGGTC CACGTG ATGATGAAGGC) (SEQ ID NO: 6) sites using the M3 IC3Δ sequence. *J. Wess (1997)*. The resulting PCR products were digested with the appropriate restriction endonucleases, purified and ligated into appropriate sites in p426GPD. Plasmids were confirmed by restriction endonuclease mapping and DNA sequencing. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, incorporated herein by reference, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9.

Yeast cells containing the MARs were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples (10^{-1} - 10^{-8} M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD₆₂₀ using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM and were plotted vs. agonist concentration. As shown in Figure 1, the yeast cells containing the M3 MAR IC3Δ produced an agonist-dependent growth response, demonstrating that

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the M3 MAR IC3Δ is functional, while the wild type MAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth. The growth response of the M3 MAR IC3Δ containing cells was dose-dependent giving an EC₅₀ for carbachol (CCh) equal to 3 μM. This value matches the K_D for CCh obtained in HEK cells (7.9 μM) and the EC₅₀ for CCh induced IP₃ (inositol triphosphate) accumulation (4.0 μM), suggesting that the M3 MAR IC3Δ retains the expected pharmacological properties when expressed in yeast cell membranes. Further, the growth response is blocked by the MAR-specific antagonist, atropine (At).

Alternatively, the response to CCh by yeast cells containing the M3 MAR IC3Δ may be observed by measuring the agonist-induced increase in fluorescent emission from a green fluorescent protein reporter gene whose expression is stimulated by MAR agonists. Green fluorescent protein (GFP) is a protein from *Aequorea* that is intrinsically fluorescent when expressed in yeast cells. The fluorescence from GFP is detectable in live yeast cells, making it possible to measure the level of its expression without any deleterious treatment of the yeast cells. This feature is particularly advantageous in the reporter gene assays that do not require additional steps to permit its detection. An inducible reporter gene that is useful in detecting the agonist-activation of heterologous GPCRs expressed in yeast utilizes transcriptional promoters that are activated by the mating signal transduction pathway. One such promoter is the *FUS2* promoter. In the absence of agonist stimulation, little or no expression of the *Fus2* protein or any other protein whose expression is directed by the *FUS2* promoter is detectable. After treatment with agonist, transcription from the *FUS2* promoter is induced up to 700 fold, leading to substantial increases in *Fus2* expression or in the expression of any gene product whose expression is placed under control of the *FUS2* promoter. Thus, yeast cell fluorescence resulting from GFP expression under the control of the *FUS2* promoter from a *FUS2*-GFP reporter gene is only observed after agonist activation of a heterologous GPCR.

In order to produce a GFP reporter gene, DNA sequences encoding the enhanced GFP (EGFP, Clontech), *FUS2* promoter and *FUS2* transcriptional terminator sequences were amplified by PCR. The fragments were assembled into the centromere containing plasmid pRS414 so as to place EGFP expression under control

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of the pheromone responsive *FUS2* promoter in the centromere containing plasmid pRS414, producing plasmid pMP241. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188, that are useful for performing assays of GPCR agonist-stimulated growth of cells containing the M3 MAR IC3Δ.

Yeast cells containing the M3 MAR IC3Δ and the *FUS2*-EGFP reporter plasmid were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2-ml SC-glucose-ura medium. The cells were washed and diluted 5 fold in SC-glucose-ura-his, pH 6.8 medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples (10^{-1} - 10^{-8} M) of CCh. The plates were incubated at 30°C for 6 hours with agitation (600 rpm). Stimulation of the *FUS2*-EGFP reporter gene expression was monitored by recording increases in emission at 530 nm after excitation with 480 nm light using a fluorescence microplate reader. Assays were conducted in duplicate and measurements obtained during the logarithmic phase of yeast cell growth. Fluorescence emission measurements were analyzed using GraphPad Prism and were presented as the mean ± SEM and were plotted vs. agonist concentration. As shown in Figure 1B, the yeast cells containing the M3 MAR IC3Δ produced a dose dependent increase in fluorescence emission, consistent with increased expression of the EGFP from the agonist inducible *FUS2*-GFP reporter gene construct. The EC50 for CCh stimulation of fluorescence emission is 4 μM, identical to values obtained in the growth assay.

Thus, deletion of a portion of the IC3 of the rat M3 MAR has produced a functional GPCR when expressed in yeast, suggesting that modification of internal domains may be a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

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Example 2. Functional Expression of a Mutated *D. melanogaster* Muscarinic Acetylcholine Receptor in Yeast

Agonist of the G protein-coupled insect muscarinic acetylcholine receptors (MARs) possess substantial insecticidal and miticidal activity. *M.R. Dick et al. (1997)*. These observations suggest that development of a yeast-based high throughput screen (HTS) for agonists active at insect MARs may be useful in identifying lead compounds that might be developed into insecticides with novel mode of action. Preliminary experiments indicate that the wild type *D. melanogaster* MAR (DMAR), an insect G protein-coupled receptors (GPCRs), is non-functional in yeast. Thus, an effort to develop a method for improving the function of the DMAR in yeast was mounted, via replacement of the DMAR IC3 with the functional IC3 from the M3 MAR IC3Δ.

In insect cells, the DMAR interacts with the heterotrimeric Gq protein leading to an increase in intracellular calcium in response to muscarinic agonists. One potential explanation for the inactivity of the DMAR in yeast is an inability to efficiently couple to the yeast heterotrimeric G protein. Thus, to devise a method to improve the DMAR function in yeast, selected mutations in the GPCRs that serve to improve functional expression and coupling to the heterotrimeric G protein were examined.

In order to construct the IC3 replacement, PCR fragments encoding three domains were prepared by standard means. Fragment 1 consisted of the amino terminal coding portion of the *Drosophila* MAR up to an *AgeI* site within the 5th TMD, amplified by PCR using oligonucleotides (AAA AGATCT AAA ATG TACGGAAACCAGACGAAC) (SEQ ID NO: 7) and (CCA GTA GAG GAA GCACATGATGGTC AGGCCT AAG TAG AAG GCG GCC AGT GC) (SEQ ID NO: 8). The second fragment of the DMAR was composed of carboxy terminal coding sequences starting with a *PmII* site in the 6th TMD, amplified by PCR using oligonucleotides (TTCATCATCACGTGGACTCCGTACAACATC) (SEQ ID NO: 9) and (AAA CTCGAG TTATCTAATTGTAGACGCGGC) (SEQ ID NO: 10). The M3 MAR IC3Δ domain was amplified as an *AgeI*-*PmII* fragment with coding sequence in frame with fragments 1 and 2, described in Example 1. These fragments were

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assembled in plasmid p426GPD to place the mutated DMAR under control of the GPD promoter. The wild type DMAR was cloned into the expression vector, pMP3, described in United States Patent 5,691,188. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188 that are useful for performing assays of GPCR agonist-stimulated growth, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9.

Yeast cells containing the DMAR and the plasmid containing the wild type DMAR were assayed in liquid culture using the MAR agonist carbachol (CCh). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8, medium containing 5 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μ l of serially-diluted samples (10^{-1} - 10^{-8} M) of the muscarinic receptor agonists. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean \pm SEM and were plotted vs. agonist concentration. As shown in Figure 2, the yeast cells containing the mutated DMAR, i.e., the M3 MAR IC3 Δ , produced an agonist-dependent growth response, demonstrating that the DMAR-M3 MAR IC3 Δ is functional. The wild type DMAR is non-functional, as indicated by the lack of agonist-dependent yeast cell growth.

Example 3. Functional Expression of a Mutated Rat Cholecystokinin CCKB Receptor in Yeast

As shown in Examples 1 and 2, deletion of portion of the IC3 of mammalian MARs, in particular the rat M3 MAR, is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. In order to test the possibility that this IC3 Δ mutation would also improve functional expression of other GPCRs in yeast, the DNA sequences

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encoding the rat wild type and IC3Δ cholecystokinin CCKB receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The wild type CCKBR was amplified by PCR using oligonucleotides

5 (ACTTAGATCAAAAAATGGAGCGCTCAAGCTGAACCG) (SEQ ID NO: 11) and (TCCCGTCGACTCAGCCAGGCCCCAGTGTGCTG) (SEQ ID NO: 12). The IC3Δ cholecystokinin CCKB receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 22 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides

10 (GGCCAGGATCCAAAAATGGGCTCCCTGCAGCCGGACGC) (SEQ ID NO: 13) and (CGGGCCCCGCGGGCGCTCGGGGCCAGACCGTTGGGC) (SEQ ID NO: 14). Fragment 2 contained carboxy terminal coding sequences including 22 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides

15 (CGGGCGACAGCCTGCCGCGGC) (SEQ ID NO: 15) and (AGCGGTCGACTCACACGATCCGCTTCCTGTCCCC) (SEQ ID NO: 16). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length CCKB receptor. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in

20 United States Patent 5,691,188, including, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9..

Yeast strains containing wild type and IC3Δ cholecystokinin CCKB receptor were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil (SCD-ura) medium. In this agar-based plate bioassay, molten

25 (50°C) SCD-ura-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH₄OH prior to autoclaving) containing 0.5 mM AT (3-aminotriazole) was inoculated with the overnight culture (2 x 10⁴ cells/ml) and poured into square (9 x 9 cm) petri plates. Solutions of CCK agonists in DMSO (1 mM, 10 μl) were applied to the surface of the solidified agar (Upper left: CCK8S; upper right, CCK8US; lower left, CCK5; lower right, CCK4). Compounds applied to the surface

30 of the plate diffused radially from the site of application and bound to CCK receptors

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expressed on the surface of cells embedded in the agar, resulting in induction of *FUS1-HIS3* expression. The responding cells formed a dense growth zone readily detectable over the limited growth of cells observed in response to basal *FUS1-HIS3* expression. Plates were incubated at 30°C for 3 days (Figure 3). Figure 3A demonstrates the robust growth response of yeast cells containing the IC3Δ cholecystokinin CCKB receptor, while Figure 3B shows only limited growth by yeast cells containing the wild type CCKB receptor, indicating that the deletion of portion of the third intracellular loop of the CCKB receptor improves its function in yeast.

Example 4. Functional Expression of a Mutated Rat Somatostatin Receptor (SSTR) in Yeast

The third intracellular loop participates in many GPCR functions, including G protein coupling, desensitization and interaction with diverse modifying proteins. Somatostatin receptors are encoded in five subtypes, labeled SSTR1-5. Several amino acids are found in the third intracellular loop of the SSTR3 subtype, but not in the equivalent region of SSTR2 subtype. Since SSTR2 functions efficiently in yeast, deletion of those amino acids from IC3 may impart this functional efficiency upon SSTR3. Thus, 8 amino acids, Gln-Trp-Val-Gln-Ala-Pro-Ala-Cys (SEQ ID NO: 17), were deleted from the third intracellular loop of the rSSTR3 cDNA, enabling more efficient receptor signaling in yeast.

Rat SSTR3 sequences were amplified by PCR using oligonucleotides containing 5' BglII and 3' XhoI sites. The resulting PCR product of approximately 1.3 kb was digested with BglII and XhoI, purified and inserted between the BamHI and XhoI sites in p426GPD to generate the plasmid p426GPD-rSSTR3. Recombinant plasmids were confirmed by restriction endonuclease digestion and DNA sequencing.

Standard PCR reactions were used to amplify the rSSTR3 cDNA to yield two PCR fragments that have 36 bp overlap as follows. PCR insert A of approximate size 750 bp was generated using the 5' Bgl oligonucleotide (AAAAAGATCT AAAATGGCCA CTGTTACCTA T) (SEQ ID NO: 18) and the 3' oligonucleotide CTCAGAGCGG CGTCGCCGCT GACACGAGGG CGCCCG (SEQ ID NO: 19).

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PCR insert B of approximate size 530 bp was generated using the 5' oligonucleotide GCGCCCTCGT GTCAGCGGCG ACGCCGCTCT GAG (SEQ ID NO: 20) and the 3' *XhoI* oligonucleotide (AAAACTCGAG TTACAGATGG CTCAGTGTGC T) (SEQ ID NO: 21). PCR fragments A and B were gel purified, annealed and amplified by PCR using the flanking 5' *BglII* and 3' *XhoI* oligonucleotides to yield the approximately 1.3 kb rSSTR3 Δ IC3 PCR product. Following purification and digestion with *BglII*-*XhoI*, the rSSTR3 Δ IC3 insert was ligated into *BamHI*-*XhoI* sites of p426GPD to generate the plasmid p426GPD-rSSTR3 Δ IC3. Restriction mapping and DNA sequencing confirmed correct reading frame and sequence.

Yeast cells of the type useful for expression of GPCRs, described in United States Patent 5,691,188, specifically, the MPY578fc cells described in *Pausch et al.* (1998) and Table 3 of Example 9, were transformed with p426GPD-rSSTR3 and p426GPD-rSSTR3 Δ IC3, using standard procedures. The cells were assayed using the agar-based bioassay format described in Example 3. Samples (10 μ l) of Somatostatin (S-14, 1mM) were applied to the surface of the selective agar medium containing the yeast cells expressing the SSTR3. The plates were incubated for 3 days at 30°C. Yeast cells transformed with p426GPD-rSSTR3 along with pLP82 (containing a G α i2/G β 12 chimeric G-protein expression plasmid) showed a weak growth response to S-14 (Figure 4A), whereas a much stronger response was observed when p426GPD-rSSTR3 Δ IC3 was assayed under similar conditions (Figure 4B). These results indicate that deletion of a portion of the IC3 improves the function of the SSTR3 in yeast.

Example 5. An IC3 deleted Human Alpha2A Adrenergic Receptor and Constitutively Active Mutant Thereof Functionally Expressed in Yeast

As shown in Examples 1-4, deletion of portion of the IC3 of mammalian GPCRs is correlated with improved functional expression in mammalian and yeast cells with retention of full ability to couple to the heterotrimeric G protein. The mutated MARs, CCKBR, and SSTR3 retain all external loops. Transmembrane domains and internal domains other than the IC3 are unchanged. The IC3, found

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between 5th and 6th membrane spanning helices, was the only domain modified. The bulk of this domain was deleted leaving only 22 amino acids proximal to both the 5th and 6th transmembrane helices. Thus, IC3 of the GPCRs containing the IC3 deletion (IC3Δ) is 44 amino acids in length. The improvement in functional expression may be due to elimination of domains known to interact with cellular desensitization mechanisms, allowing more functional MAR to be retained at the cell surface.

In order to test the possibility that other IC3Δ mutations would also improve functional expression of other GPCRs in yeast, DNA sequences encoding an IC3Δ human alpha2A adrenergic receptor were amplified by PCR and cloned into proximity to the glycerol-phosphate dehydrogenase promoter in yeast expression plasmid, p426GPD, by standard methods. The IC3Δ human alpha2A adrenergic receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 39 amino acids proximal to the 5th TMD, amplified by PCR using oligonucleotides (GGCCAGGATCCAAAAATGGGCTCCCTGCAGCCGGACGC) (SEQ ID NO: 13) and (CGGGCCCCGCGGGCGCTCGGGGCCAGACCGTTGGGC) (SEQ ID NO: 14). Fragment 2 contained carboxy terminal coding sequences including 41 amino acids proximal to the 6th TMD, amplified by PCR using oligonucleotides (CGGGCGACAGCCTGCCGCGGC) (SEQ ID NO: 15) and (AGCGGTCGACTCACACGATCCGCTTCCTGTCCCC) (SEQ ID NO: 16). The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length alpha2A adrenergic receptor. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9.

In order to test the ability of constitutively active GPCRs to induce a yeast growth response in the absence of cognate agonist, a similar construct was prepared from a constitutively active mutant (CAM) human alpha2A adrenergic receptor. The CAM receptor contains a point mutation that changes threonine residue 373 to lysine (T373K), rendering the receptor highly active in the absence of agonist while retaining

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considerable agonist-inducible activity. The CAM IC3Δ human alpha2A adrenergic receptor was prepared by fusing two overlapping fragments. Fragment 1 contained amino terminal coding sequences including 38 amino acids proximal to the 5th TMD. Fragment 2 contained carboxy terminal coding sequences including 33 amino acids proximal to the 6th TMD. The two fragments were fused by amplification by PCR using oligos at 5' and 3' ends of the full length alpha2A adrenergic receptor. Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9.

Yeast cells containing the wild type IC3Δ and CAM IC3Δ human alpha2A adrenergic receptor were assayed in liquid culture using the alpha adrenergic receptor full agonist UK14304 (RBI) and partial agonist clonidine. The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium. Samples of the cell suspension (180 μl) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μl of serially-diluted samples of the adrenergic receptor agonist, UK14304 (10^{-3} - 10^{-10} M) and partial agonist clonidine (10^{-3} - 10^{-9} M). The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD620 using a microplate reader. Assays were conducted in duplicate and growth rate measurements obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean ± SEM and were plotted vs. agonist concentration.

The yeast cells containing the wild type IC3Δ and CAM IC3Δ human alpha 2A adrenergic receptor produced a dose-dependent growth response, indicating that this IC3 deletion is functional (Figure 5). Interestingly, at UK14304 levels too low to produce an agonist-dependent growth response, the basal growth rate of the CAM receptor-containing cells is greater than that of the cells containing the wild type receptor, indicating that the CAM receptor is more active in the absence of agonist. In Figure 6, in the presence of 5mM AT, a concentration sufficient to eliminate the basal growth of both the wild type IC3Δ and CAM mutant-containing cells, yet permitting a

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UK14304 inducible growth response (Figure 6A), only the CAM IC3Δ human alpha2A adrenergic receptor is able to produce a growth response to the partial agonist clonidine (Figure 6B), consistent with elevated activity of the CAM receptor. For both UK14304 and clonidine, the ED50s are shifted to lower agonist concentrations in the CAM receptor containing strains, consistent with the expected activity of a CAM human alpha2A adrenergic receptor. The growth responses were reversed by the adrenergic antagonist yohimbine.

The IC3Δ α2A adrenergic receptor was found to be functional, indicating that modification of the third intracellular loop in the manner described results in a functional GPCR. The CAM version of the IC3Δ α2A adrenergic receptor possessed the capacity to induce yeast cell growth independent of applied agonist, suggesting that the constitutive activity of any CAM GPCR may be measured simply by assessing the level of yeast cell growth that occurs in the absence of agonist. This effect of CAMs in GPCRs may be particularly useful in the testing of orphan GPCRs, for which no cognate agonist is known. Thus, an orphan GPCR may be mutated in its third intracellular loop to make it a CAM GPCR and tested for its ability to stimulate a yeast growth response in the absence of agonist. If the CAM orphan GPCR is capable of stimulating a yeast cell growth response, then it must be functional and therefore suitable for inclusion in a drug screening assay.

Example 6. Expression of a CAM M3 Muscarinic Acetylcholine Receptor (M3 MAR) in Yeast

In Example 5, a CAMα2A adrenergic receptor was shown to be able to stimulate a yeast cell growth response in the absence of added agonist. The CAM was in the third intracellular loop, a domain known to be involved in both activation of GPCRs and in interaction with G proteins. The second intracellular loop and regions proximal to it is also known to be involved in GPCR activation. Within this region, a conserved tripeptide motif, Asp-Arg-Tyr, known as the DRY box, has been shown to be involved in activation of rhodopsin family GPCRs. Site-directed mutants of the α1-adrenergic receptor in which the Asp residue was modified to hydrophobic amino

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acids, including Ile, were correlated with high constitutive activity. A CAM M5-MAR produced by mutation of a site in sixth transmembrane domain proximal the carboxy terminus of the third intracellular loop was evaluated in mammalian cell culture. *Spauldin et al. (1995)*. The elevated basal signaling due to the CAM was reversed by a variety of inverse agonists, including atropine, confirming the increased intrinsic activity of the CAM M5-MAR.

In order to test the effect of a DRY box mutation in a GPCR of known function, the rat M3 MAR was modified to replace the Asp residue in the DRY box with an Ile residue. The rat M3-MAR yeast expression plasmid described in Example 1 was used as template for the D (Asp) to I (Ile) change in the DRY box, which was accomplished by site-directed mutagenesis using a Stratagene QuikChange kit according to manufacturers instructions. Yeast cells competent to functionally express GPCRs of the kind described in United States Patent 5,691,188, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9, were transformed with the modified M3 MAR expression plasmid using a conventional lithium acetate procedure.

The growth responses of yeast cells containing the DRY box mutated M3-MAR expression plasmid were compared to those containing the unmodified M3-MAR in the agar-based bioassay format. Yeast cells were cultured overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil (SCD-ura) medium. In this agar-based plate bioassay, molten (50°C) SCD-ura-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated KOH prior to autoclaving) containing 0.5 mM AT was inoculated with the overnight culture (2×10^4 cells/ml) and poured into square (9x9 cm) petri plates. Solutions of the MAR agonist carbachol (10 mM, 10 μ l) and the inverse agonist, atropine (1 mM or 10 mM, 10 μ l) were applied to the surface of the plate. Compounds applied to the surface of the plate diffused radially from the site of application and bound to MAR receptors expressed on the surface of cells embedded in the agar, resulting in either agonist induction of *FUS1-HIS3* expression or growth inhibition by the inverse agonist. The plates were incubated at 30°C for 3 days.

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5 The CAM M3-MAR promotes an elevated yeast cell growth rate in the absence of agonist which may be observed by comparison of the areas of the plate that were not treated with either agonist or antagonist with similar portions of the non-CAM M3-MAR plate. That the elevated growth response is due to a CAM M3-MAR is confirmed by the reversal of agonist-independent growth by the inverse agonist, atropine (Figure 7A). Atropine had no toxic effect on the growth yeast cells expressing the CAM M3-MAR when grown in the presence of histidine. The non-CAM M3-MAR retained the ability to be agonist-stimulated, while the CAM M3-MAR was no longer responsive to agonist.

10 These results indicate that introduction of CAMs into the DRY box of GPCRs results in a measurable increase in agonist-independent yeast cell growth due to its elevated constitutive activity. The increased growth rate indicates the mutated GPCR is functionally active in the absence of agonist. This effect of DRY box CAMs in GPCRs may be particularly useful in the testing of orphan GPCRs, since the DRY box is widely conserved and thus, should be present in most orphan receptors. Thus, an orphan GPCR may be mutated in its DRY box to make it a CAM GPCR and tested for its ability to stimulate a yeast growth response in the absence of agonist. If the CAM orphan GPCR is capable of stimulating a yeast cell growth response, then it must be functional and therefore suitable for inclusion in a drug screening assay.

20 **Example 7. Truncation of the Rat Neurotensin NT1 Receptor Causes an Increase in Agonist Sensitivity**

25 In examples 1-4, modification of the third intracellular loop leads to improvement in functional expression of a variety of heterologous GPCRs expressed in yeast. Agonist induced desensitization of the GPCRs is also mediated in part by GPCR internal domains other than the third intracellular loop, such as the intracellular carboxy-terminal tail.

30 Elimination of the carboxy terminal domains from GPCRs has been shown to improve functional expression in yeast and mammalian cells. Truncation of the carboxy terminal tail of the G protein-coupled alpha mating pheromone receptor

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expressed in α -mating type yeast cells causes supersensitivity to the presence of mating pheromone (*Reneke et al. (1988)*; *Konopka et al (1988)*). Consistent with these observations, a mutated rat neurotensin NT1 receptor (rNTR1) lacking its carboxy terminal tail is resistant to agonist-induced internalization when expressed in mammalian cells (*Hermans et al. (1996)*).

To test whether carboxy-terminal truncation improves the functional response of a heterologous GPCR expressed in yeast, the rat NTR1 was modified by deleting the 52 amino acids that constitute the carboxy terminal tail, leaving a shortened receptor 372 amino acids in length. The coding sequences of the wild type and truncated neurotensin NT1 receptor (rNTR1 C-term Δ), were amplified by PCR using a 5' oligonucleotide primer that specified a common amino-terminal coding sequence (AGTCAGATCTAAGCTT AAAA ATG CAC CTC AAC AGC TCC) (SEQ ID NO: 22) and separate oligos that define the wild type (AGTC AGATCT CTA GTA CAG GGTCTCCC) (SEQ ID NO: 23) and truncated carboxy termini (AGAG AGATCT TTAGCGCCACCCAGGACAAAGGC) (SEQ ID NO: 24). These fragments were cloned into proximity of the PGK promoter in the yeast expression vector pPGK by standard methods (*Y-S. Kang et al. (1990)*). Using a conventional lithium acetate transformation procedure, the resulting plasmids were introduced into yeast cells of the kind described in United States Patent 5,691,188, specifically, the MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9 that are useful for performing assays of GPCR agonist-stimulated growth.

Yeast cells containing the NTR1s were assayed in liquid culture using the NT receptor agonist acetyl neurotensin 8-13 (AcNt8-13). The cells were cultured overnight in 2 ml SC-glucose-ura medium. The cells were diluted 500 fold in SC-glucose-ura-his, pH 6.8 medium containing 2 mM 3-aminotriazole to decrease basal growth rate. Samples of the cell suspension (180 μ l) were dispensed to wells of sterile 96 well microtiter dishes containing 20 μ l of serially-diluted samples (10^{-3} - 10^{-10} M) of AcNt8-13. The plates were incubated at 30°C for 18 hours with agitation (600 rpm). Growth was monitored by recording increases in OD₆₂₀ using a microplate reader. Growth rate measurements were obtained during the logarithmic phase of

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yeast cell growth. Optical density measurements were analyzed using GraphPad Prism and are presented as the mean \pm SEM and were plotted against agonist concentration. As shown in Figure 8, the yeast cells containing the NTR1s produced an agonist-dependent growth response demonstrating that both the wildtype and carboxy terminally truncated NTR1s were functional. The growth response of the rNTR1 C-term Δ containing cells was dose-dependent giving an EC₅₀ for AcNT8-13 equal to 520 nM. This value is five fold lower than observed for cells expressing the wildtype NTR1 (2.1 μ M). The carboxy terminal deletion has produced a rNTR1 that responds to a lower concentration of NTR agonist improving the sensitivity of the yeast bioassay.

Thus, deletion of a portion of the carboxy terminal intracellular domain of the rat NTR1 has produced a functional GPCR with increased agonist sensitivity when expressed in yeast, suggesting that modification of this internal domain is a generalizable method for improving the function of heterologous GPCRs expressed in yeast.

Example 8. Production of Yeast Sterol Mutants

To demonstrate that yeast sterol mutants will increase the efficiency of G protein-coupled receptor signaling, heterologous G protein-coupled receptors were expressed in *erg6* mutants constructed from MPY578fc giving an agonist-dependent growth response. (*M. Pausch et al. (1998)*). The strain was constructed by transformation of MPY578fc (*M. Pausch et al., (1998)*) with DNA containing an *erg6* deletion cassette which deletes the endogenous ERG6 gene. The cassette is constructed by using the following DNA oligonucleotides as primers in a PCR reaction:

5' ATGAGTGAAACAGAATTGAGAAAAAGACAGGCCCAATTCAGTAG
GGAGTTACATGGTGATTTGTCACCTTACGTACAATC 3' (SEQ ID NO:
25) and

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5'

TCGTGCGCTTTATTTGAATCTTATTGATCTAGTGAATTTATTGAGTT
GCTTCTTGGGAAGGGCAAGTGCACAAACAATAC 3' (SEQ ID NO: 26).

This PCR reaction using plasmid pRS404 (Stratagene company) DNA results in the synthesis of a DNA fragment containing sequences homologous to the 5' and 3' sequences surrounding the *ERG6* gene but with the entire *TRP1* gene intervening and replacing *ERG6* sequences. The strain that results from the transformation of the cassette is named YML103.

To test the extent of signal transduction of a heterologously expressed GPCR, a number of plasmid constructs containing such receptors were transformed into strain MPY578fc cells described in *Pausch et al. (1998)* and Table 3 of Example 9 and into strain YML103. GPCR genes, including the human V1a and V2 vasopressin receptors, the human melanocortin receptor (MC4), the rat somatostatin SSTR2 receptor, the rat M3 MAR and the rat CCKB receptor, were inserted into plasmid p426GPD in the manner described in the preceding examples. The V1a and V2 receptor-transformed strains demonstrate vasopressin-dependent growth responses in the *erg6*-deleted YML103 strain. Such a response is not detected in the wild type MPY578fc strain (Figure 9A-D). With the rat somatostatin SSTR2 receptor, the rat M3 MAR receptor and the rat CCKB receptor transformed strains, the signal to noise ratio (agonist induced growth compared to the growth in the background of the plate where agonist is not present) is increased, leading to a clearer signal (Figure 10 A-B).

Example 9. Functional activity of heterologous GPCRs expressed in yeast strains containing chimeric G alpha proteins.

Yeast cells containing various chimeric G alpha proteins were rendered dependent on agonist-stimulated activation of coexpressed heterologous GPCRs and downstream elements of the pheromone response pathway for growth on selective medium. The technology was exploited to examine aspects of ligand-receptor and receptor-G protein interactions.

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Plasmid constructions

The rat somatostatin SSTR2 and rat adenosine A2a receptor expression plasmids (pJH2, pLP110) were described elsewhere (*L.A. Price et al. (1995)*, *L.A. Price et al. (1996)*). The protein-coding region of the rat NTR1 (*K. Tanaka et al. (1990)*) was modified by PCR for efficient expression in yeast. Oligonucleotides that add a 5' *Hind*III site and a yeast translational initiation consensus sequence to the 5' end (TCTC AAGCTT AAAA ATG CGC CTC AAC AGC TCC GCG)(SEQ ID NO: 27) and a *Bgl*II site to the 3' end after the termination codon (ACAC AGATCT CTA GTA CAG CGT CTC GCG GG)(SEQ ID NO: 28) were used to amplify the NTR1 sequences. The resulting fragment was purified, digested with the appropriate restriction endonucleases and inserted between *Hind*III and *Bam*HI sites in the phospho-glycerate kinase (PGK1) promoter containing expression vector, pPGKH (*Y.S. Kang et al. (1990)*), forming pMP198.

The protein-coding region of the human MCR4 (*I. Gantz et al. (1993)*) was modified by PCR for efficient expression in yeast. Oligonucleotides that add a 5' *Hind*III site and a yeast translational initiation consensus sequence to the 5' end (TCTC AAGCTT AAAA ATG CGC CTC AAC AGC TCC GCG)(SEQ ID NO: 27) and a *Bgl*II site to the 3' end after the termination codon (ACAC AGATCT CTA GTA CAG CGT CTC GCG GG)(SEQ ID NO: 28) were used to amplify the MCR4 sequences. The resulting fragment was purified, digested with the appropriate restriction endonucleases and inserted between *Hind*III and *Bam*HI sites in the phospho-glycerate kinase (PGK1) promoter containing expression vector, pPGKH (*Y.S. Kang et al. (1990)*), forming pMP223. The wild type rat CCK-B-R expression plasmid pMP203 was constructed by amplifying the protein-coding region of the rat CCK-B-R (*S.A. Wank et al. (1992)*) by PCR. Oligonucleotides that add a 5' *Hind*III site and a yeast translational initiation consensus sequence to the 5' end (ATTT AAGCTT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 29) and a *Bgl*II site to the 3' end after the termination codon (TCCC AGATCT TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 30) were used to amplify the rat CCK-B-R sequences. The resulting fragment was purified, digested with the appropriate restriction endonucleases and inserted between *Hind*III and

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*Bam*HI sites in the phosphoglycerate kinase (PGK1) promoter containing expression vector, pPGKH (*Y.S. Kang et al.*, (1990)), forming pMP203.

A second rat CCK-B-R expression plasmid, pMP258, was constructed using oligos that add a 5' *Bgl*II site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *Sal*I site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG) (SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al.* (1995)).

The protein-coding region of the human CCK-A receptor (*A. de Weerth et al.* (1993)) was modified by PCR for efficient expression in yeast. Oligonucleotides that add a *Hind*III site and a yeast translational initiation consensus sequence to the 5' end (AAAA AAGCTT AAAA ATG GAT GTG GTT GAC AGC CTT)(SEQ ID NO: 32) and a *Bgl*II site to the 3' end after the termination codon (AAAA AGATCT TCA GAC CCC ACC GTG GCT)(SEQ ID NO: 33) were used to amplify the CCK-A receptor sequences. The resulting fragment was purified, digested with the appropriate restriction endonucleases and inserted between *Hind*III and *Bam*HI sites in the phospho-glycerate kinase (PGK1) promoter containing expression vector, pPGKH (*Y.S. Kang et al.* (1990)), forming pMP209.

A rat M3-muscarinic acetylcholine receptor (*T.I. Bonner et al.* (1987)) expression plasmid, pEK290, was constructed using oligos that add a 5' *Bgl*II site and a yeast translational initiation consensus sequence to the 5' oligo (GTCA AGATCT AAAA ATG ACC TTG CAC AGT AAC)(SEQ ID NO: 34). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *Xho*I site (TACC CTCGAG CTA CAA GGC CTG CTC CGG C)(SEQ ID NO: 35). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al.* (1995)).

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A *Drosophila melanogaster* muscarinic acetylcholine receptor (*R. A. Shapiro et al. (1989)*) expression plasmid, pEK289, was constructed using oligos that add a 5' *Bgl*III site and a yeast translational initiation consensus sequence to the 5' oligo (ATCC AGATCT AAAA ATG TAC GGA AAC CAG ACG AAC GG)(SEQ ID NO: 36). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *Xho*I site (TAAG CTCGAG TTA TCT AAT TGT AGA CGC GGC G)(SEQ ID NO: 37). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*).

A human GnRH receptor (*S.S. Kakar et al. (1992)*) expression plasmid, p426GPD-hGnRHR, was constructed using oligos that add a 5' *Bgl*III site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *Sal*I site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*).

A human vasopressin V2 receptor (*M. Birnbaumer et al. (1992)*) expression plasmid, p426GPD-hV2R, was constructed using oligos that add a 5' *Bgl*III site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *Sal*I site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*). A human histamine H3 receptor (*T. W. Lovenberg et al. (1999)*) expression plasmid, pET19, was constructed using oligos that add a 5' *Bgl*III site and a

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yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *SaII* site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*).

A human adrenergic α 2A receptor (*C.A. Guyer et al. (1990)*) expression plasmid, pMP249, was constructed using oligos that add a 5' *Bgl*II site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *SaII* site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*).

A human β 2-adrenergic receptor (*B.K. Kobilka et al. (1987)*) expression plasmid, pET19, was constructed using oligos that add a 5' *Bgl*II site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination codon by adding a *SaII* site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*K. Tanaka et al. (1990)*).

An *Aplysia californica* octopamine OA1 receptor (*E. Kandel, Columbia University, personal communication*) expression plasmid, pMP255, was constructed using oligos that add a 5' *Bgl*II site and a yeast translational initiation consensus sequence to the 5' oligo (ATTT AGA TCT AAAA ATG GAG CTG CTC AAG CTG AAC CG)(SEQ ID NO: 31). The 3' oligonucleotide specified the 3' end after the termination

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codon by adding a *SalI* site (TCCC GTC GAC TCA GCC AGG CCC CAG TGT GCTG)(SEQ ID NO: 12). The fragment obtained using the above oligos was purified, digested with the appropriate restriction endonucleases and inserted between *Bam*HI and *Xho*I sites in the glycerolphosphate dehydrogenase (GPD) promoter containing expression vector p426GPD (*D. Mumberg et al. (1995)*).

A vector suitable for replacing the chromosomal *GPA1* sequences with chimeric genes encoding the mammalian C-terminal amino acids was produced. The base vector was produced as follows. A fragment of the *GPA1* gene (*C. Dietzel et al. (1987)*, *I. Miyajima et al. (1987)*, *M. Nakafuku et al. (1987)*) comprising the promoter and coding sequence was isolated from pPGKH-SCG1 (*Y.S. Kang et al. (1990)*). The silent mutation that formed the *Bam*HI site was placed within the *GPA1* open reading frame at a position at which a conserved *Bam*HI site is often found in G protein alpha subunit coding sequences. One 0.5 kb *GPA1* fragment was amplified from sequences composed of the 3' regulatory sequences using oligos that added *Bam*HI and *Xba*I (AAA GGATCC AGG AAC TGT ATA ATT AAA GTA (SEQ ID NO: 38); and ATG TCTAGA AAT TAA CAA CAA TAA AGA)(SEQ ID NO: 39). A second 0.5 kb *GPA1* fragment with *Xba*I and *Sal*I sites (ATT TCTAGA CAT TGT TTC ATT AAT TGA (SEQ ID NO: 40); and TTT GTCGAC TTA TCT CAT CAC TGG CAT TTA)(SEQ ID NO: 41) was produced. The fragments were inserted sequentially into corresponding sites in YIp5 (*M.F. Rose et al. (1990)*), a URA3-containing integrating vector, forming pLP136. The resulting construct may be linearized using the artificial *Xba*I site inserted between 3' fragments.

Chimeric G protein integrating vectors were constructed by adding to pLP136 *Bam*HI fragments encoding the carboxy terminus of *Gpa1* with the 5 carboxy terminal amino acids of mammalian G proteins. The *Bam*HI fragments were amplified using oligos that placed *Bam*HI sites in frame at both ends as indicated below in Table 1.

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Table 1. Oligonucleotides used to construct with chimeric *GPA1* alleles containing modified carboxy termini coding sequences.

5' GPA1: 5' AAG TGG ATC CAT TGT TTC GAA GGA ATT ACA G (SEQ ID NO: 42)

$\alpha_t, \alpha_{i1}, \alpha_{i2}$: 5' AGC TGG ATC CTC AAA ACA AAC CAC AAT CTT TAA GGT TTT GCTGGA TGA TTA G (SEQ ID NO: 43)

α_{i3} : 5' AGC TGG ATC CTC AAA ACA AAC CAC ATT CTT TAA GGT TTT GCT GGATGA TTA G (SEQ ID NO: 44)

α_o : 5' AGC TGG ATC CTC AAT ACA AAC CAC AAC CTT TAA GGT TTT GCT GGATGA TTA G (SEQ ID NO: 45)

α_s : 5' AGC TGG ATC CTC ACA ACA ATT CAT ATT GTT TAA GGT TTT GCTGGA TGATTA G (SEQ ID NO: 46)

α_z : 5' AGC TGG ATC CTC AAC ACA AAC CAA TAT ATT TAA GGT TTT GCT GGA TGATTA G (SEQ ID NO: 47)

$\alpha_{q,11}$: 5' AGC TGG ATC CTC AAA CCA AAT TAT ATT CTT TAA GGT TTT GCT GGA TGA TTA G (SEQ ID NO: 48)

α_{12} : 5' AGC TGG ATC CTC ATT GCA ACA TAA TAT CTT TAA GGT TTT GCT GGATGA TTA G (SEQ ID NO: 49)

α_{13} : 5' AGC TGG ATC CTC ATT CCA ACA TCA ATT GTT TAA GGT TTT GCT GGATGA TTA G (SEQ ID NO: 50)

α_{14} : 5' AGC TGG ATC CTC AAA CCA AAT TAA ATT CTT TAA GGT TTT GCT GGA TGA TTA G (SEQ ID NO: 51)

$\alpha_{15,16}$: 5' AGC TGG ATC CTC ACA ACA AAT TAA TTT CTT TAA GGT TTT GCT GGA TGA TTA G (SEQ ID NO: 52)

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The 3' oligos specified the coding sequences for the 5 carboxy-terminal amino acids from each of the mammalian G α proteins. The fragments were digested with *Bam*HI and inserted in the correct orientation into the sole *Bam*HI site in pLP136, producing the integrating plasmids identified below in Table 2.

Table 2. Plasmids used to replace the *GPA1* gene with chimeric GPA1 alleles containing modified carboxy termini coding sequences.

	<u>Plasmid</u>	<u>G protein alpha subunits</u>	<u>Carboxy-terminal amino acids</u>
		Gpa1	KIGII (SEQ ID NO: 53)
1)	pGPA1-Gai2	Gat, Gai1, Gai2	DCGLF (SEQ ID NO: 54)
2)	pGPA1-Gai3	Gai3	ECGLF (SEQ ID NO: 55)
3)	pGPA1-Gao	Gao1, Gao2	GCGLY (SEQ ID NO: 56)
4)	pGPA1-Gaz	Gaz	YIGLC (SEQ ID NO: 57)
5)	pGPA1-Gaq	Gaq, Gal1	EYNL (SEQ ID NO: 58)
6)	pGPA1-Ga12	Ga12	DIMLQ (SEQ ID NO: 59)
7)	pGPA1-Ga13	Ga13	QLMLE (SEQ ID NO: 60)
8)	pGPA1-Ga14	Ga14	ENFLV (SEQ ID NO: 61)
9)	pGPA1-Ga16	Ga15, Ga16	EINLL (SEQ ID NO: 62)
10)	pGPA1-Gas	Gas1, Gas2	QYELL (SEQ ID NO: 63)

DNA fragments encoding the carboxy termini of mammalian G alpha proteins were obtained from existing expression plasmids (*Y.S. Kang et al. (1990)*). The fragments were inserted in the correct orientation into the sole *Bam*HI site in pLP136.

A *FUS1*-LacZ reporter gene bearing plasmid (pMP283) was constructed by transferring the *Pst*I-SalI *FUS1*-LacZ fragment from pSL307 (*G. McCaffrey et al. (1987)*) to corresponding sites in the multicopy TRP1 containing vector, pRS426 (*T.W. Christianson et al.*).

Strain constructions

Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedure (*M.F. Rose et al. (1990)*). Yeast strains

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containing chimeric G proteins were constructed by two step integrative replacement of the *GPA1* locus. In order to target this construct to integrate at the *GPA1* locus, the chimeric G protein expression plasmids were digested with *XbaI*. DNA-mediated transformation of MPY578fc cells to uracil prototrophy using the linear fragment was carried out using the lithium acetate method (A. St. Jean *et al.* (1991)). Selection for replacement of the *GPA1* locus was accomplished by plating on FOA medium (M.F. Rose *et al.* (1990)). Yeast cells surviving this procedure were examined by PCR to demonstrate the presence of the altered *GPA1* allele.

In order to construct yeast shuttle vectors containing chimeric GPA1 genes, a 3' fragment of the GPA1 gene was amplified using the oligonucleotides shown in Table 1. The 3' coding fragment contained coding sequences for the carboxy-terminal GPCR binding domain of GPA1 in which the five carboxy-terminal amino acids were replaced with those of mammalian G alpha proteins. The resulting fragments were subcloned into pLP136, a *URA3*, integrating shuttle vector based on YIp5 (M.F. Rose *et al.* (1990)). The resulting constructs were linearized with *XbaI* to promote integration at the *GPA1* locus and used to transform MPY578fc cells (M.H. Pausch *et al.* (1998)), resulting in the yeast strains identified below in Table 3.

Table 3. Yeast strains used in this study

Strain Genotype

MPY578fc	<i>MATa GPA1 ste2ΔLEU2 far1ΔLYS2 sst2ΔSST2-NEO fus1ΔFUS1-HIS3 fus2ΔFUS2CAN1 ura3 leu2 trp1 his3 lys2 ade2 can1⁺</i>
MPY578αi2	MPY578fc <i>gpa1ΔGPA1-Gαi2</i>
MPY578αi3	MPY578fc <i>gpa1ΔGPA1-Gαi3</i>
MPY578αo	MPY578fc <i>gpa1ΔGPA1-Gαo</i>
MPY578αq	MPY578fc <i>gpa1ΔGPA1-Gαq</i>
MPY578αs	MPY578fc <i>gpa1ΔGPA1-Gαs</i>
MPY578αz	MPY578fc <i>gpa1ΔGPA1-Gαz</i>
MPY578α12	MPY578fc <i>gpa1ΔGPA1-Gα12</i>
MPY578α13	MPY578fc <i>gpa1ΔGPA1-Gα13</i>

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MPY578 α 14 MPY578fc *gpa1* Δ *GPA1-G α 14*MPY578 α 16 MPY578fc *gpa1* Δ *GPA1-G α 16*

5 The Ura⁺ transformants were passaged on FOA medium and replacement of the *GPA1* locus with the chimeric alleles confirmed. The resulting strains (Table 3) were then transformed with plasmids conferring expression of various heterologous GPCRs. The yeast strains were assayed in the agar or liquid formats using appropriate cognate agonists. The ability of each GPCR/G protein alpha subunit strain to transduce a growth-promoting signal was determined and the results displayed in Figure 11.

10 The GPCRs assayed in yeast displayed G protein alpha subunit coupling selectivity expected from their behavior in their native contexts. For example, the neurotensin NTR1, a Gq coupled receptor in mammalian cells (*K. Tanaka et al. (1990)*), promotes growth in Gpa1-Gq/11 containing yeast strains, while failing to promote growth in Gpa1-Gs strains. NTR1 promotes growth in the Gpa1-G16 strain, as expected given the promiscuous coupling behavior of G16 in mammalian cells (*G. Milligan et al. (1996)*). Conversely, the vasopressin V2 receptor, a Gs coupled receptor in mammalian cells (*M. Birnbaumer et al. (1992)*), promotes growth in Gpa1-Gs containing yeast strains, while failing to promote growth in Gpa1-Gq/11 strains. Taken together, these data indicate that GPCRs expressed in yeast retain G protein alpha subunit coupling selectivity. Thus, the yeast strains described here represent a convenient tool useful for demonstrating the G protein alpha subunit coupling selectivity of GPCRs.

20
25 **Example 10. Agonist-independent growth stimulated by the adenosine A2a receptor overexpressed in yeast strains containing chimeric G alpha proteins.**

30 In order to employ orphan GPCRs in HTS assays, functional activity of the GPCRs must be demonstrated in the absence of agonist. In the above examples, mutations introduced into GPCRs confer a constitutively activated state, which leads to an increase yeast cell growth rate in the absence of agonist. An alternative approach arises from the observation that constitutive activation of downstream

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signaling pathways results when certain wild-type GPCRs are overexpressed in mammalian cells. This approach leads to constitutive activity and activation of downstream signaling pathways in the absence of agonist.

The phenomenon may be explained by increased accumulation of GPCRs that have switched spontaneously to the active state. In the simple three-state model that describes GPCR activation, the receptor exists in equilibrium between a unique state R, the active state R*, and the inactive state R° states, with the inactive state predominant in the absence of agonist. The GPCR may switch spontaneously between R and the active and inactive configurations. Agonists stabilize the active configuration, permitting GPCRs to stimulate downstream signaling molecules. At wildtype expression levels, spontaneous conversion to the active configuration does not permit accumulation of GPCRs to a level that is sufficient to produce detectable activation of downstream signaling pathways above basal levels. When GPCRs are overexpressed, more of the receptors are present and capable of transforming spontaneously into the active configuration, thus activating downstream signaling pathways by mass action.

Application of this approach in the yeast cell expression system described herein suggests that overexpression of GPCRs should lead to activation of growth in the absence of agonist. This growth activation should be proportional to the amount of receptor expressed; more receptors expressed leads to a faster growth rate in the absence of agonist. As well, growth activation should be observed only in the cells in which GPCR and G protein are able to couple efficiently.

To test this approach, rat adenosine A2a receptor expression plasmids conferring high (pLP116, a multicopy plasmid, described in *Price L. A., Strnad J., Pausch M. H., and Hadcock, J. R.*, Pharmacological characterization of the rat A2a adenosine receptor functionally coupled to the yeast pheromone response pathway, *Mol. Pharmacol.* 50, 829-837, 1996) and low (pMP145, a low-copy number, centromere containing plasmid, described in *Pausch et al.*) levels of receptor were introduced into yeast cells useful for performing assays of GPCR agonist-stimulated growth, such as those described in United States Patent 5,691,188. Specifically,

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MPY578fc cells described in *Pausch et al.* (*Pausch, M. H., Price, L. A., Kajkowski, E. M., Strnad, J., Delacruz, F., Heinrich, J. A., Ozenberger, B. A., and Hadcock, J. R.*, Heterologous G protein-coupled receptors in *Saccharomyces cerevisiae*, Methods for genetic analysis and ligand identification, *Identification and expression of G protein-coupled receptors*, *Lynch, K. R.*, Ed., Wiley-Liss, New York, NY, 1998, 196-212) and the chimeric G protein containing strains described in Example 9 were transformed with pLP116, pMP145 and the empty vector pRS426 using the conventional lithium acetate transformation.

MPY578fc and related chimeric G protein containing strains expressing the adenosine A2a receptor were assayed in liquid culture. Eight individual transformants of each plasmid-containing strain were cultured overnight in 0.2 ml SC-glucose-ura medium. The cells were diluted 500 fold into 0.2 ml cultures of SC-glucose-ura-his, pH 6.8 medium containing 2, 5 or 10 mM 3-aminotriazole in 96 well plates. Adenosine deaminase is added (0.13 mg/ml) to degrade and inactivate any adenosine produced by the yeast cells. Growth was monitored by recording increases in OD570 using a microplate reader. Growth rate measurements were obtained during the logarithmic phase of yeast cell growth. Optical density measurements were analyzed using GraphPad Prism. As shown in Figure 11, the yeast cells containing the adenosine A2a receptor produced an agonist-independent growth response, demonstrating that the functional activity of the adenosine A2a receptor may be detected in the absence of agonist. As expected, the adenosine A2a receptor produced the highest growth rate when expressed at high levels in cells which contain G alpha proteins to which it is able to couple efficiently, e. g. Gpa1 and Gpa1-s5 and Gpa1- α 16. The agonist-independent growth response is also proportional to the amount of adenosine A2a receptor produced, i. e. cells containing the multicopy adenosine A2a receptor expression plasmid, pLP116, grew faster than cells containing the low copy number plasmid, pMP145.

These results indicate that the functional activity of orphan GPCRs may be detected in the absence of agonist by overexpression in yeast cells that contain various chimeric G protein constructs. Thus, an orphan GPCR may be overexpressed in yeast

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cells that contain various chimeric G protein constructs in the absence of agonist. If the orphan GPCR is capable of stimulating yeast cell growth, and does so selectively via coupling to a subset of chimeric G proteins, then it must be function and, therefore, is suitable for inclusion in a drug screening assay. Such screening assay would be performed under empirically determined receptor expression condition in which the stimulatory effect of agonist would be detected over the basal growth rate.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

The references cited herein are specifically incorporated by reference in their entirety.

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We claim:

1. A host cell comprising a constitutively active heterologous G protein-coupled receptor.
2. The host cell according to claim 1, wherein the host cell is a eukaryotic cell.
3. The host cell according to claim 2, wherein the heterologous G protein-coupled receptor is modified at an intracellular domain of the G protein-coupled receptor.
4. The host cell according to claim 3, wherein the intracellular domain is the third intracellular loop.
5. The host cell according to claim 2, wherein the host cell is yeast.
6. The host cell according to any one of claims 1 to 5, wherein the heterologous G protein-coupled receptor is an orphan receptor.
7. The host cell according to claim 5, wherein the heterologous G protein-coupled receptor is modified at amino acid residues Asp-Arg-Tyr in the domain proximal to the second intracellular loop of the G protein-coupled receptor.
8. The host cell according to claim 5, wherein the modified G protein-coupled receptor is a human alpha 2A adrenergic receptor and the modification comprises a point mutation of threonine to lysine at amino acid residue 373.
9. The host cell according to claim 8, wherein the modification further comprises a truncated third intracellular loop having 44 amino acids.
10. The host cell according to claim 7, wherein the heterologous G protein-coupled receptor is a M3 muscarinic acetylcholine receptor.
11. The host cell according to claim 10, wherein the aspartic acid residue is replaced by a hydrophobic amino acid.
12. The host cell according to claim 11, wherein the hydrophobic amino acid is isoleucine.

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13. A method for screening compounds capable of binding to G protein-coupled receptors comprising the steps of (a) subjecting the host cell according to claim 1 to a test compound; and (b) measuring the effect of the test compound on cell growth.

14. The method according to claim 13, wherein the host cell is yeast.

15. A host cell comprising a heterologous G protein-coupled receptor, and a mutation of a host cell gene that results in an improved functional response of the G protein coupled receptor in a cell-based assay.

16. The host cell according to claim 15, wherein the mutation results in improved agonist stimulated growth promoting ability.

17. The host cell according to claim 15, wherein the mutation results in improved coupling between the heterologous G protein-coupled receptor and a heterotrimeric G protein or failure of the receptor to interact with cell desensitization or sequestration-internalization machinery, or proper plasma membrane localization.

18. The host cell according to claim 15, wherein the host cell gene encodes a regulatory receptor protein kinase, and the mutation causes a reduction in receptor phosphorylation.

19. The host cell according to claim 18, wherein the regulatory receptor protein kinase is selected from the group consisting of G protein-coupled receptor kinases, protein kinase A, protein kinase C and casein kinase.

20. The host cell according to claim 15, wherein the host cell gene encodes a component of the endocytic or degradative pathway and the mutation causes a reduction in receptor sequestration, internalization, or degradation.

21. The host cell according to claim 15, wherein the mutation affects the ratio or nature of sterols in the host cell membrane.

22. The host cell according to claim 21, wherein the host cell is yeast and the host cell gene is selected from the group consisting of *ERG2*, *ERG3*, *ERG4*, *ERG5*, and *ERG6*.

23. The host cell according to claim 22, wherein the host cell gene is *ERG6*.

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24. The host cell according to claim 23, wherein the heterologous G protein-coupled receptor is selected from the group consisting of a human melanocortin receptor, a rat somatostatin SSTR2 receptor, a rat M3 muscarinic acetylcholine receptor, and a rat CCKB receptor.

25. The host cell according to claim 21, wherein the host cell is yeast and the host cell gene is selected from the group consisting of *HEM1*, *HEM3*, *SUT1*, *PDX3*, *UPC1*, and *UPC2* (*UPC20*) and wherein the mutation allows the host cell to grow in the presence of exogenously added sterols.

26. A method for screening compounds capable of binding to G protein-coupled receptors comprising the steps of (a) subjecting the host cell according to claims 15, 18, 20, or 21 to a test compound; and (b) measuring the effect of the test compound on cell growth.

27. A method for expressing constitutively active heterologous G protein-coupled receptors in a host cell comprising:

- (a) transforming the host cell with a vector comprising a DNA sequence encoding a modified heterologous G protein-coupled receptor, wherein the modification results in a constitutively active G protein-coupled receptor; and
- (b) culturing the transformed host cell to permit expression of the heterologous G protein-coupled receptor.

28. The method according to claim 27, wherein the host cell is yeast.

29. A host cell comprising a modified G protein alpha subunit gene, wherein the modified G protein alpha subunit gene encodes a chimeric G alpha protein.

30. The host cell according to claim 29, wherein the host cell is a eukaryotic cell.

31. The host cell according to claim 30, wherein the host cell is yeast.

32. The host cell according to claim 29, wherein the modified G protein alpha subunit gene comprises a first nucleic acid sequence encoding the amino terminal domain of an endogenous G alpha protein, linked to a second nucleic acid sequence encoding the carboxy terminus of a heterologous G alpha protein.

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33. The host cell according to claim 29, wherein the modified G protein alpha subunit gene comprises a substitution of a first nucleic acid sequence encoding the five carboxy terminal amino acids of an endogenous G alpha protein for a second nucleic acid sequence encoding the five carboxy terminal amino acid sequences of a heterologous G alpha protein.

34. The host cell according to claim 32, wherein the amino terminal domain of the G alpha protein comprises an interaction domain for a G beta protein, a G gamma protein, and an effector molecule.

35. The host cell according to claim 32 or 33, wherein the modified G protein alpha subunit gene is *GPA1*.

36. The host cell according to claim 32 or 33, further comprising a heterologous G protein-coupled receptor.

37. The host cell according to claim 36, wherein the modified G protein alpha subunit gene is *GPA1* and the host cell is yeast.

38. The host cell according to claim 36, wherein the heterologous G alpha protein is a mammalian protein.

39. The host cell according to claim 37, wherein the modified *GPA1* gene comprises a first nucleic acid sequence encoding the amino terminal domain of an endogenous G alpha protein, linked to a second nucleic acid sequence encoding the carboxy terminus of a mammalian G alpha protein selected from the group consisting of G*ai*2, G*ai*3, G*ao*, G*as*, G*aq*, G*az*, G*α*11, G*α*12, G*α*13, G*α*14, G*α*15, and G*α*16.

40. The host cell according to claim 37, wherein the modified *GPA1* gene comprises a substitution of a first nucleic acid sequence encoding the five carboxy terminal amino acids of an endogenous G alpha protein for a second nucleic acid sequence encoding the five carboxy terminal amino acid sequences of a mammalian G alpha protein selected from the group consisting of G*ai*2, G*ai*3, G*ao*, G*as*, G*aq*, G*α*11, G*az*, G*α*12, G*α*13, G*α*14, and G*α*15, and G*α*16.

41. The host cell according to claim 36, wherein the heterologous G protein-coupled receptor is selected from the group consisting of rat somatostatin SSTR2, rat adenosine A2a, rat muscarinic acetylcholine M2 and M3, *D. melanogaster* muscarinic

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acetylcholine M1, rat neurotensin NT-1, human vasopressin V2, rat cholecystokinin CCK-A and CCK-B, human gonadotropin releasing hormone GnRH, human melanocortin MCR4, human adrenergic α 2A, *Aplysia californica* octopamine OA1, human bombesin receptor related sequence 3 (BRS3), human histamine H3, and human β 2-adrenergic receptor.

42. An isolated DNA sequence encoding a chimeric G alpha protein, wherein the DNA sequence comprises a first nucleic acid sequence encoding the amino terminal domain of a G alpha protein of a first species, and a second nucleic acid sequence encoding the carboxy terminus of a G alpha protein of a second species, which is different from the first species.

43. The DNA sequence according to claim 42, wherein the amino terminal domain of the G alpha protein of the first species comprises an interaction domain for a G beta protein, for a G gamma protein, and for an effector molecule.

44. An isolated DNA sequence encoding a chimeric G alpha protein, wherein a first nucleic acid sequence encoding the five carboxy terminal amino acids of a G alpha protein from a first species is substituted for a second nucleic acid sequence encoding the five carboxy terminal amino acid sequences of a G alpha protein from a second species, which is different from the first species.

45. A polypeptide encoded by the DNA claim 42 or 44.

46. A method of measuring agonist-stimulated activation of a heterologous G protein-coupled receptor in a host cell comprising:

- (a) transforming the host cell according to claim 29 with a vector comprising a DNA sequence encoding a heterologous G protein-coupled receptor;
- (b) culturing the transformed host cell in the presence of an agonist specific for the heterologous G protein-coupled receptor; and
- (c) measuring the growth of the host cell in response to the agonist to determine the agonist-stimulated activation of the heterologous G protein-coupled receptor.

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47. A method of measuring the coupling specificity of a G alpha protein for a heterologous G protein-coupled receptor comprising:

- (a) transforming a host cell according to claim 29 with a vector comprising a DNA sequence encoding a heterologous G protein-coupled receptor;
- (b) culturing the transformed host cell in the presence of an agonist specific for the heterologous G protein-coupled receptor; and
- (c) measuring the growth of the host cell in response to the agonist to determine the coupling specificity of the G alpha protein for the heterologous G protein-coupled receptor.

48. The method according to claims 46 or 47, wherein the host cell is yeast.

49. A method of measuring agonist-stimulated activation of a heterologous G protein-coupled receptor in a host cell comprising:

- (a) culturing a host cell according to claim 36 in the presence of an agonist specific for the heterologous G protein-coupled receptor; and
- (b) measuring the growth of the host cell in response to the agonist to determine the

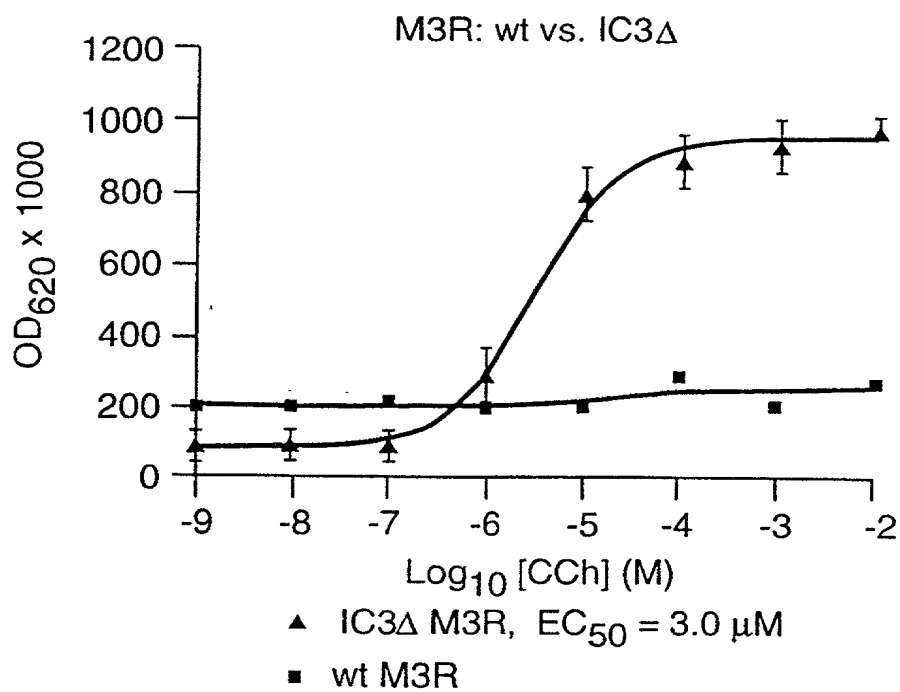
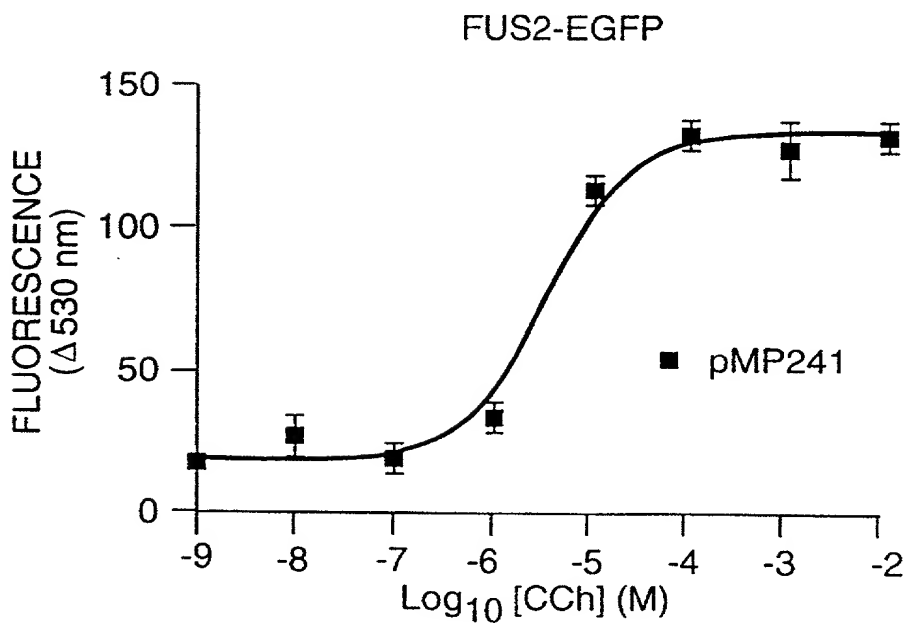
agonist-stimulated activation of the heterologous G protein-coupled receptor.

50. A method of measuring the coupling specificity of a G alpha protein for a heterologous G protein-coupled receptor comprising:

- (a) culturing a host cell according to claim 36 in the presence of an agonist specific for the heterologous G protein-coupled receptor; and
- (b) measuring the growth of the host cell in response to the agonist to determine the coupling specificity of the G alpha protein for the heterologous G protein-coupled receptor.

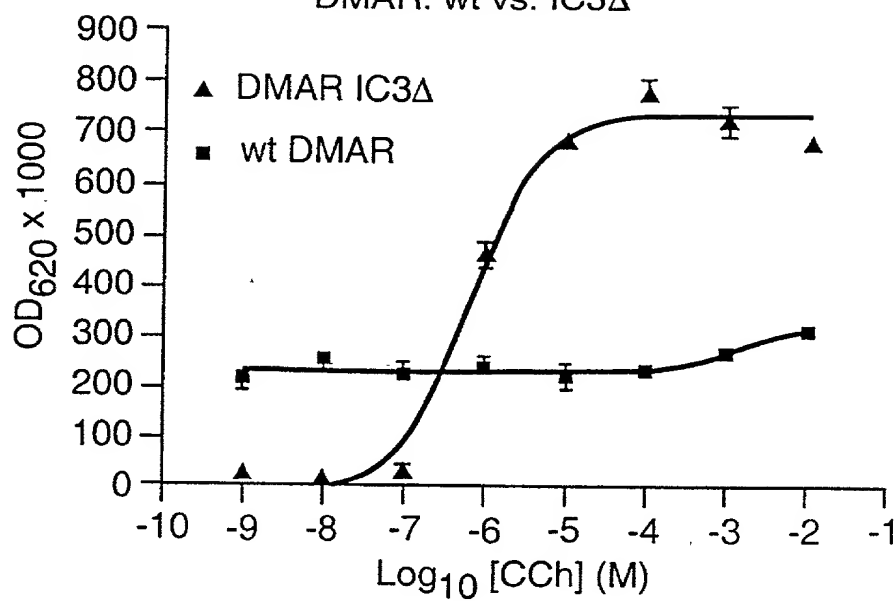
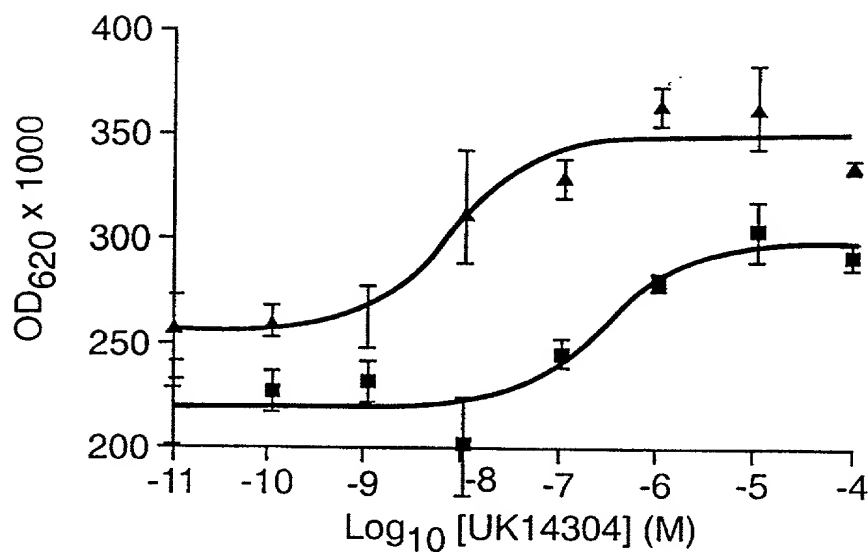
51. The method according to claim 49 or 50, wherein the host cell is yeast.

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**FIG. 1A****FIG. 1B**

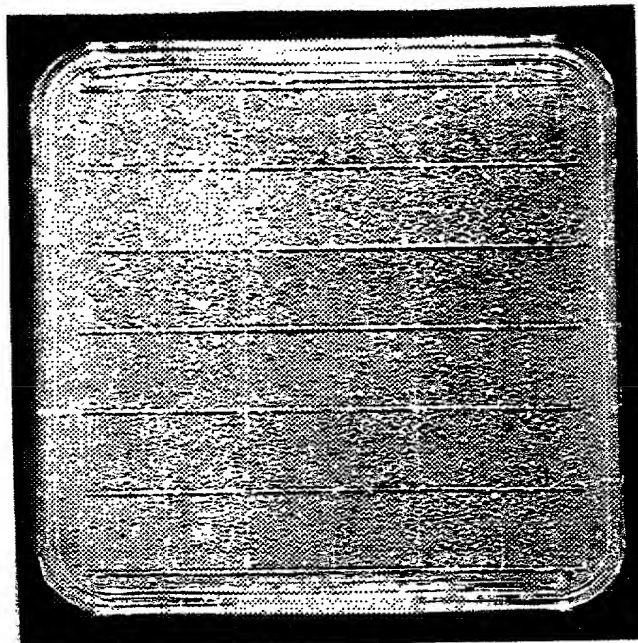
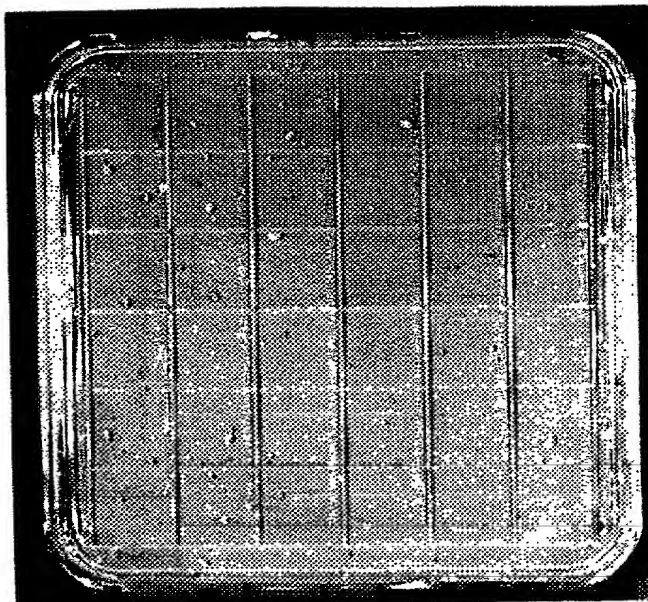
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DMAR: wt vs. IC3Δ

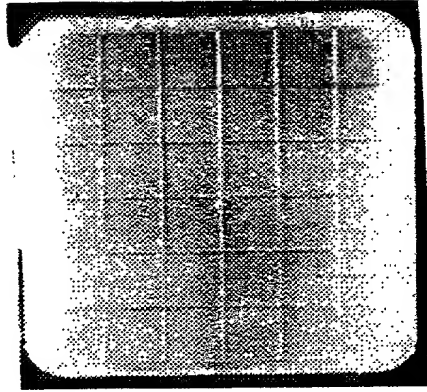
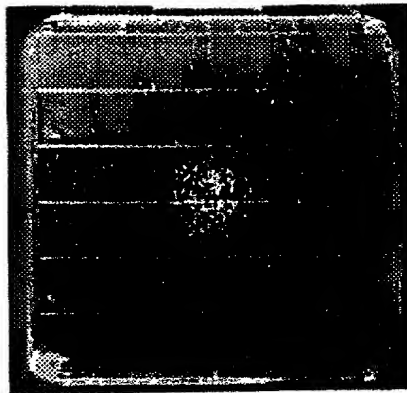
**FIG. 2** α_{2a} - ADRENERGIC RECEPTOR■ IC3Δ, EC₅₀ = 270 nM▲ IC3Δ CAM (T373K), EC₅₀ = 7.4 nM**FIG. 5**

SUBSTITUTE SHEET (RULE 26)

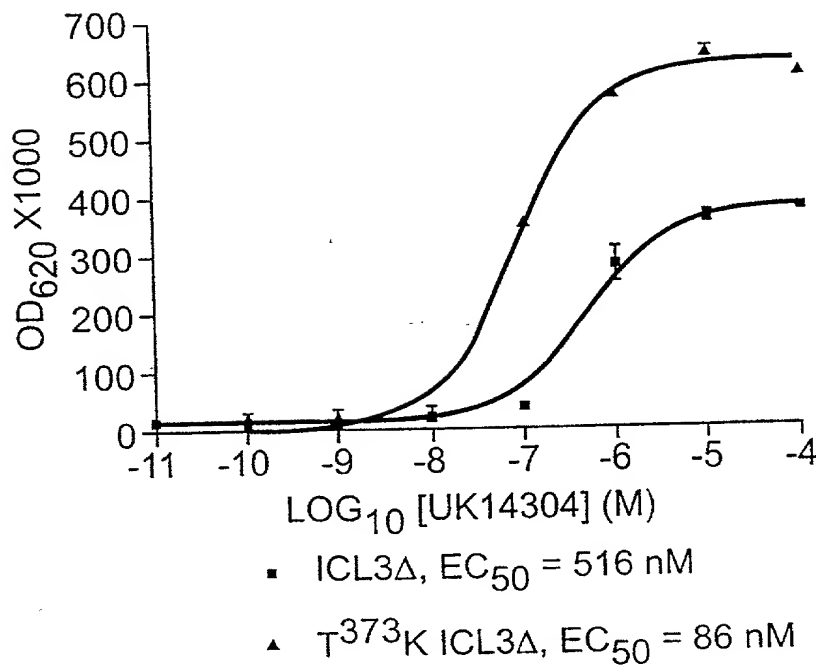
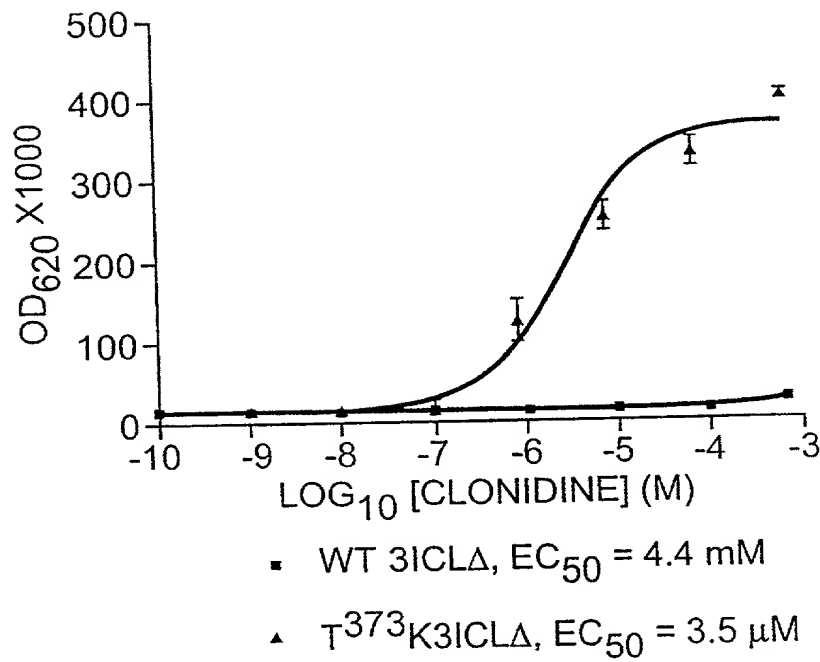
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*FIG. 3A**FIG. 3B*

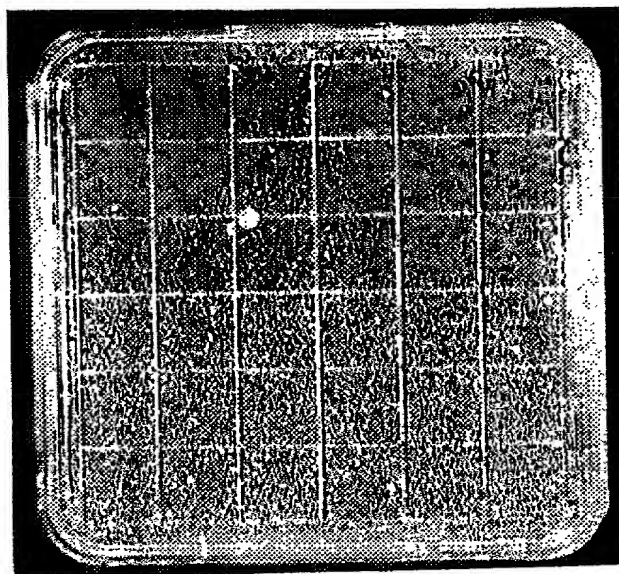
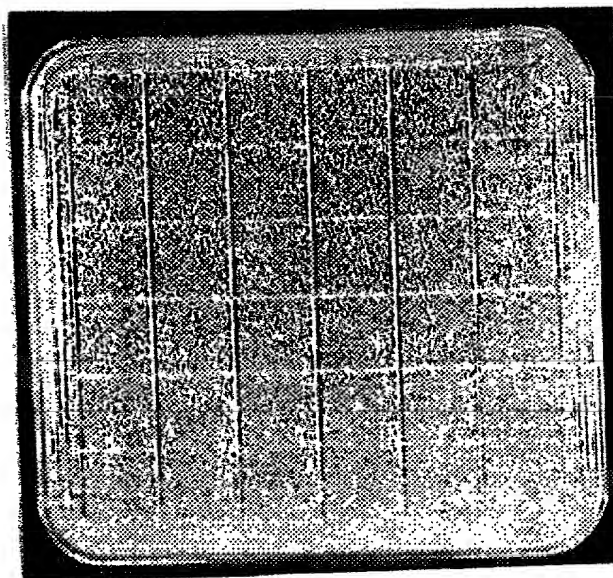
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*FIG. 4A**FIG. 4B*

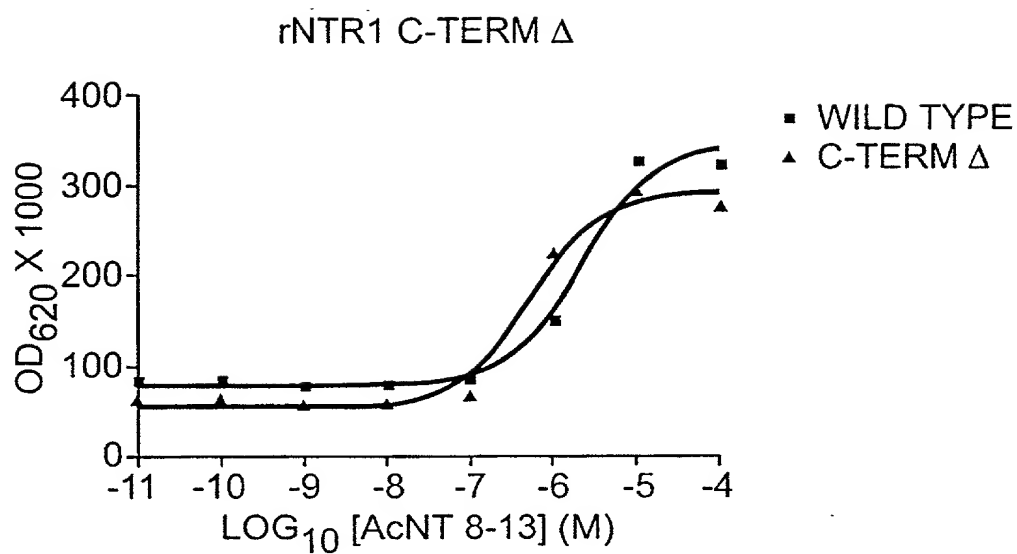
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 $h\alpha_{2a}AR$ (5 mM AT) $h\alpha_{2a}AR$ (5 mM AT)**FIG. 6**

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*FIG. 7A**FIG. 7B*

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**FIG. 8**

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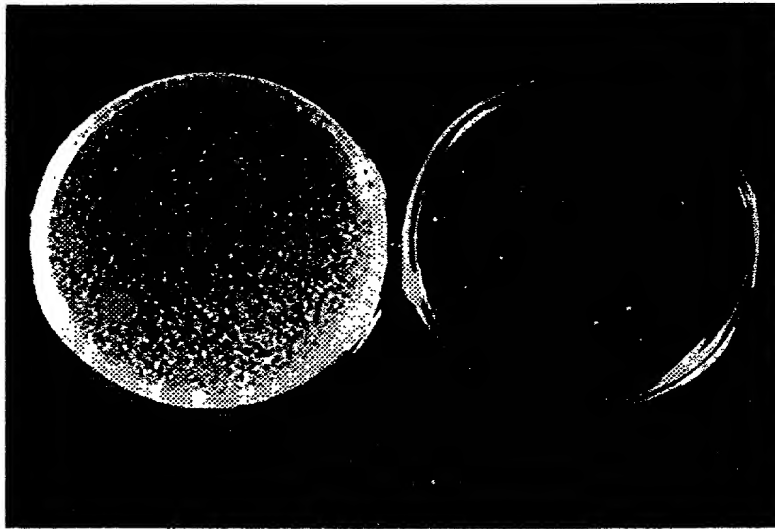


Fig. 130 Sec. B-D WP 135 G-10 Date: 04-17-1998 Time: 12:30 ID#561-0230 File: C-VMS2001 IMAGE MARGARET V28A.TIF

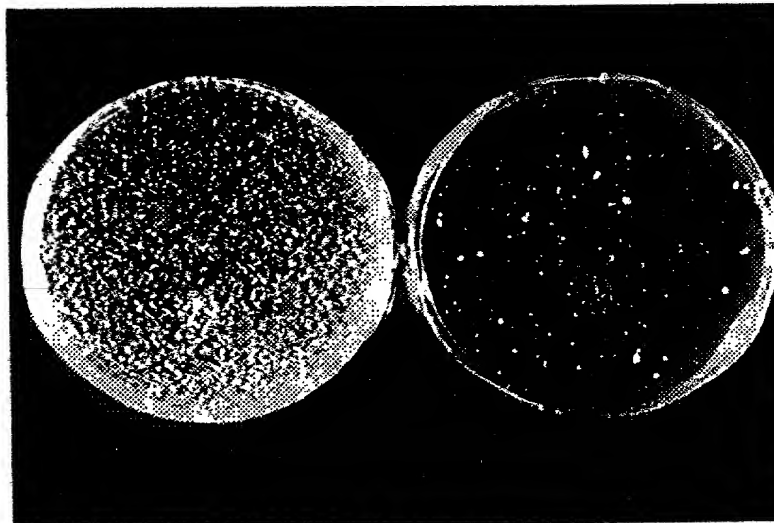
FIG. 9A

Fig. 130 Sec. B-D WP250 G-100 Date: 04-29-1998 Time: 10:31 ID#561-0230 File: C-VMS2001 IMAGE MARGARET V28A.TIF

FIG. 9B

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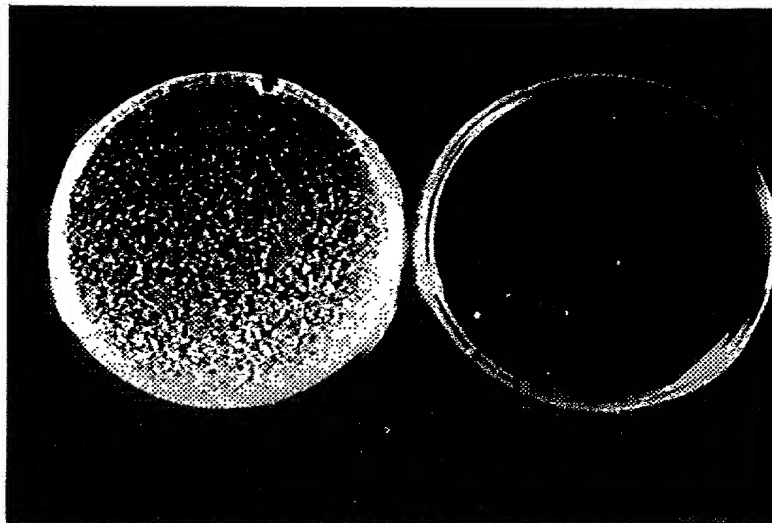


FIG. 9C

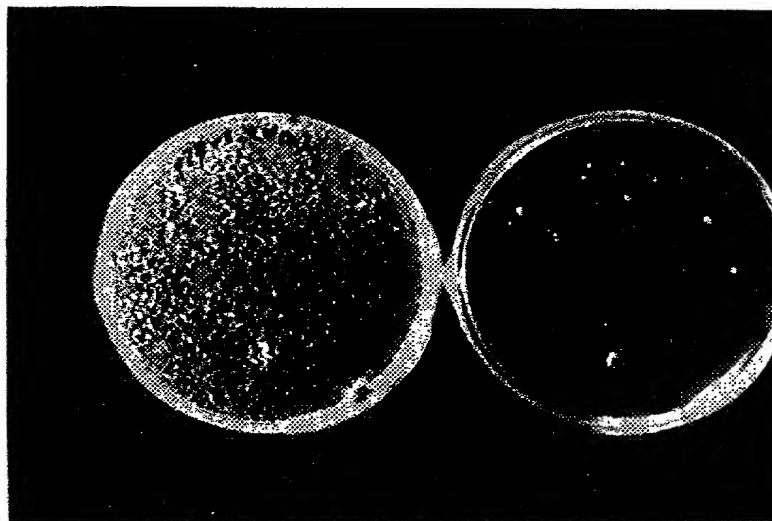


FIG. 9D

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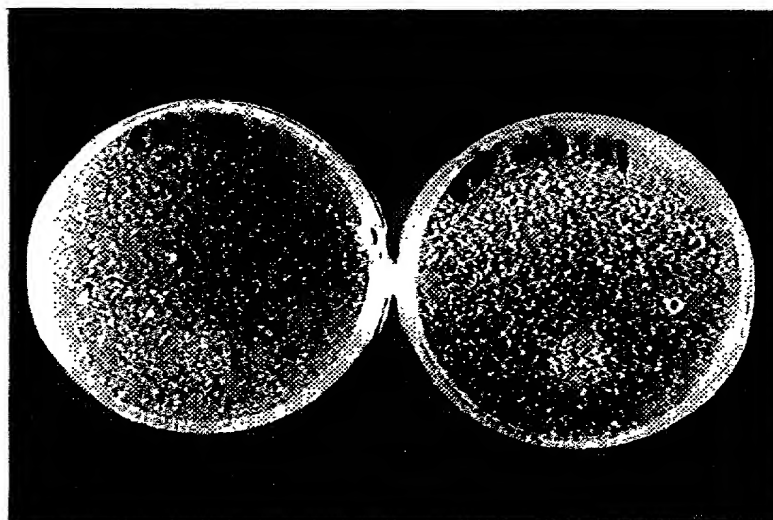


FIG. 10A

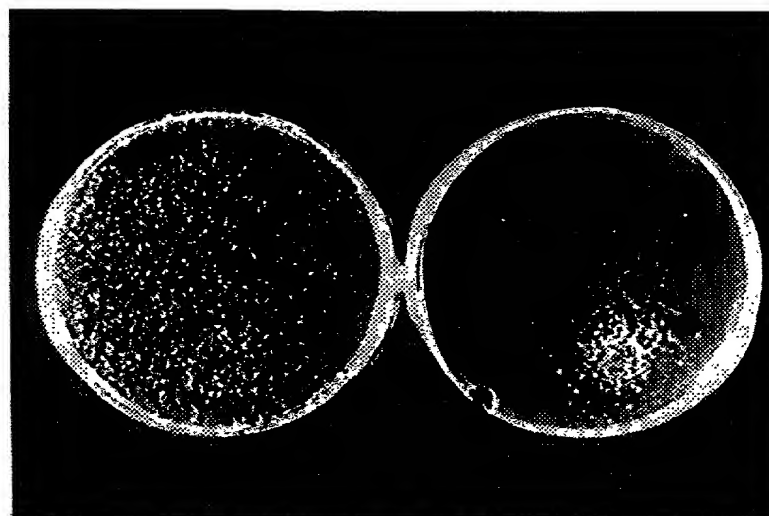


FIG. 10B

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GROWTH RESPONSES ELICITED BY VARIOUS GPCRS EXPRESSED IN CHIMERIC
G PROTEIN STRAINS

GPCR	GPA1	YEAST STRAINS CONTAINING CHIMERIC GPA1 ALLELES							
		α i2	α i3	α o	α q	α s	α z	α 12	α 13 α 14 α 16
SSTR2	+	+		+			+		+
A2aR	+					+			+
M3 MAR	-				+				+
Dm MAR	-				+				+
NTR1	+				+	-			+
MCR4	+					+			+
CCK-B-R	+				+				+
CCK-A-R	+				+				+
β 2-AR	-					+			
α 2A-AR	+	+		+			+		
AcOAR	+					+			
H3R	-	-		+			-		
GnRHR	-				+				
BRS3	-						+		
V2R	+				-	+			

FIG. 11

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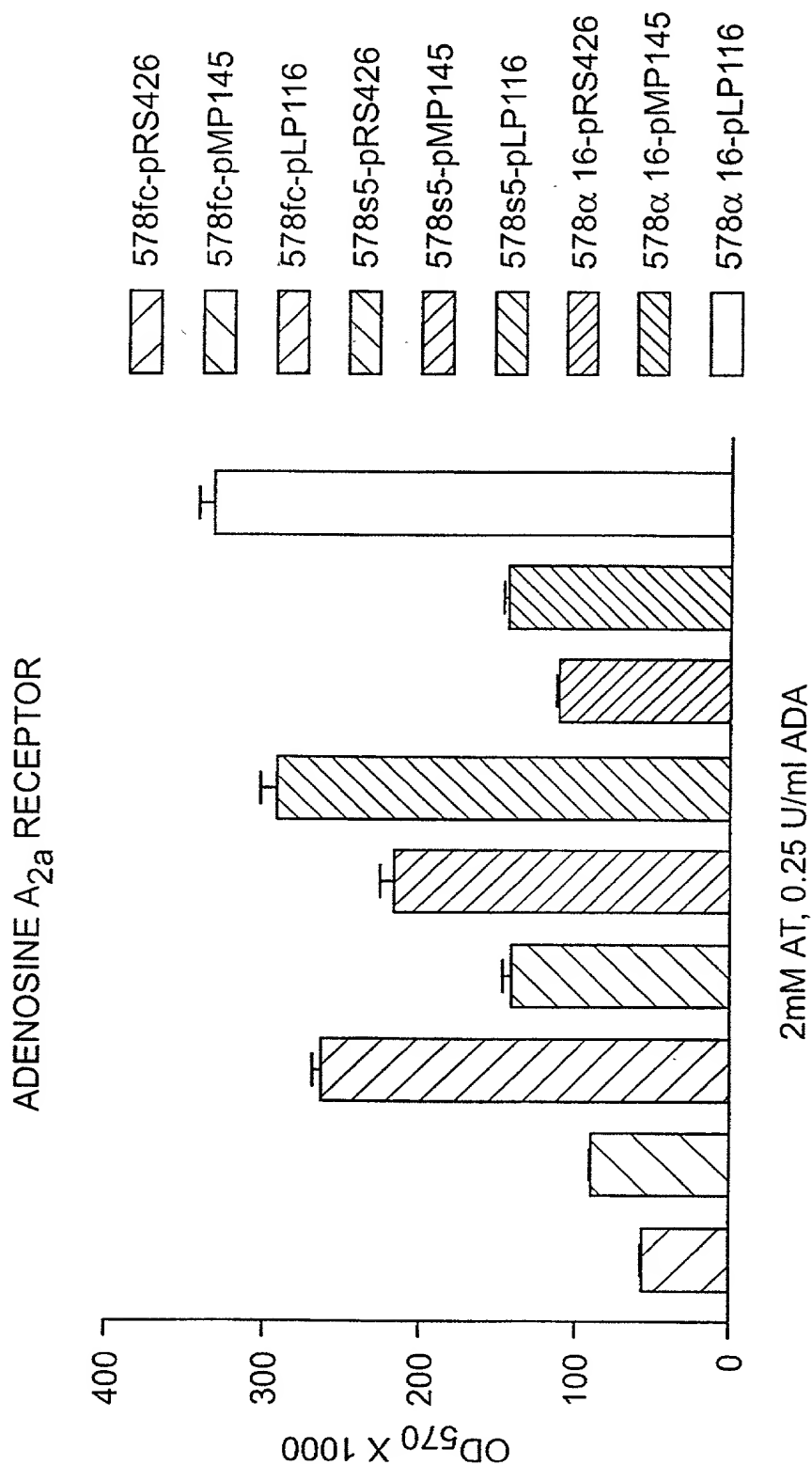


FIG. 12



Attorney Docket No. 01142.0101
Application No. 09/786,056

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am an original, first, and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ENHANCED FUNCTIONAL EXPRESSION OF G PROTEIN-COUPLED RECEPTORS

the application for which was filed on March 1, 2001, as attorney docket number 01142.0101, and which was accorded United States Application Serial No. 09/786,056. The application is the U.S. National Stage application of PCT International Application Number PCT/US99/20011, which was filed on September 1, 1999.

I hereby state that I have reviewed and understand the contents of the above-identified application, including the claims. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. § 119(a)-(d)	
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing
60/098,704	September 1, 1998

Attorney Docket No. 01142.0101

Application No. 09/786,056

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56, and which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)


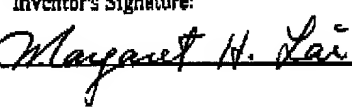
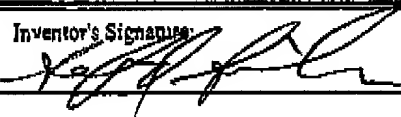
I hereby appoint the following attorneys and agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilly, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewis, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James B. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,629; Allen R. Jensen, Reg. No. 28,224; Lori Ann Johnson, Reg. No. 34,498; David A. Manspeizer, Reg. No. 37,540; Leslie A. McDonell, Reg. No. 34,872; Matthew T. Latimer, Reg. No. 44,024; Charles F. Costello, Reg. No. 27,324;

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Barbara V. Maurer, Reg. No. 31,278; Karen M. Dellerman, Reg. No. 33,692; David T. Banchik, Reg. No. 36,439; Fernando A. Borrego, Reg. No. 34,780; Mary E. Golota, Reg. No. 36,814; Anne G. Sabourin, Reg. No. 33,772; Brian W. Stegman, Reg. No. 30,977; Gerald L. Coon, Reg. No. 29,910; Mark A. Westhafer, Reg. No. 42,220; Elaine T. Sale, Reg. No. 41,286; Mary K. Cameron, Reg. No. 34,789; and Michael F. Morgan, Reg. No. 42,906.

Please address all correspondence to FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P. 1300 I Street, N.W., Washington, D.C. 20005, Telephone No. (202) 408-4000.

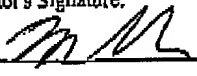
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of First Inventor: <u>Mark Henry PAUSCH</u>	Inventor's Signature: 	Date: <u>11/5/01</u>
Residence: <u>23 Montgomery Ave., Rocky Hill, New Jersey 08553</u> NJ		Citizenship: U.S.A.
Post Office Address: same as above		
Full Name of Second Inventor: <u>Margaret Hsien-fen Kuh LAI</u>	Inventor's Signature: 	Date: <u>11/2/01</u>
Residence: <u>18 Manton Ave, East Brunswick, New Jersey 08816</u> NJ		Citizenship: U.S.A.
Post Office Address: same as above		
Full Name of Third Inventor: <u>Sanford SILVERMAN</u>	Inventor's Signature: 	Date: <u>11-2-01</u>
Residence: <u>10 N. Valley Rd., Roosevelt, New Jersey 08555</u> NJ		Citizenship: U.S.A.
Post Office Address: P.O. Box 657, Roosevelt, New Jersey 08555		

Attorney Docket No. 01142,0101
 Application No. 09/786,056

Full Name of Fourth Inventor: <u>Camelia Birsan CHIRIAC</u>		Inventor's Signature: <i>Camelia Birsan</i>	Date: <u>11/2/01</u>
Residence: <u>15 Linden Ln. South, Plainsboro, New Jersey 08536 NJ</u>		Citizenship: <u>Canada</u>	
Post Office Address: <u>same as above</u>			
Full Name of Fifth Inventor: <u>William BAUMBACH</u>		Inventor's Signature:	Date:
Residence: <u>32 Loudell St, Hopewell, New Jersey 08525</u>		Citizenship: <u>U.S.A.</u>	
Post Office Address: <u>same as above</u>			
Full Name of Sixth Inventor: <u>Eugene TSENG</u>		Inventor's Signature: <i>Eugene Tseng</i>	Date: <u>11/2/01</u>
Residence: <u>232 Windmill Court, Bridgewater, New Jersey 08807 NJ</u>		Citizenship: <u>U.S.A.</u>	
Post Office Address: <u>same as above</u>			
Full Name of Seventh Inventor: <u>Eileen Marie KAJKOWSKI</u>		Inventor's Signature: <i>Eileen Marie Kajkowski</i>	Date: <u>11/2/01</u>
Residence: <u>336 Rileyville Rd, Ringoes, New Jersey 08561 NJ</u>		Citizenship: <u>U.S.A.</u>	
Post Office Address: <u>same as above</u>			
Full Name of Eighth Inventor: <u>Bradley Alton OZENBERGER</u>		Inventor's Signature: <i>Bradley Alton Ozenberger</i>	Date: <u>11/2/01</u>
Residence: <u>8 Eagleton Farms Road, Newtown, Pennsylvania 18940 PA</u>		Citizenship: <u>U.S.A.</u>	
Post Office Address: <u>same as above</u>			

Attorney Docket No. 01142.0101
Application No. 09/786,056

Full Name of Fourth Inventor: Camelia Birsan CHIRIAC	Inventor's Signature:	Date:
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Post Office Address: same as above		
Full Name of Fifth Inventor: William BAUMBACH	Inventor's Signature: 	Date: 10-31-01
Residence: 32 Louellen St., Hopewell, New Jersey 08525 NJ		Citizenship: U.S.A.
Post Office Address: same as above		
Full Name of Sixth Inventor: Eugene TSENG	Inventor's Signature:	Date:
Residence: 232 Windmill Court, Bridgewater, New Jersey 08807		Citizenship: U.S.A.
Post Office Address: same as above		
Full Name of Seventh Inventor: Eileen Marie KAJKOWSKI	Inventor's Signature:	Date:
Residence: 336 Rileyville Rd, Ringoes, New Jersey 08551		Citizenship: U.S.A.
Post Office Address: same as above		
Full Name of Eighth Inventor: Bradley Alton OZENBERGER	Inventor's Signature:	Date:
Residence: 8 Baglston Farms Road, Newtown, Pennsylvania 18940		Citizenship: U.S.A.
Post Office Address: same as above		



Attorney Docket No. 01142.0101

Application No. 09/786,056

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am an original, first, and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**ENHANCED FUNCTIONAL EXPRESSION OF
G PROTEIN-COUPLED RECEPTORS**

the application for which was filed on March 1, 2001, as attorney docket number 01142.0101, and which was accorded United States Application Serial No. 09/786,056. The application is the U.S. National Stage application of PCT International Application Number PCT/US99/20011, which was filed on September 1, 1999.

I hereby state that I have reviewed and understand the contents of the above-identified application, including the claims. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. § 119(a)-(d)	
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing
60/098,704	September 1, 1998

Attorney Docket No. 01142.0101

Application No. 09/786,056

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56, and which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorneys and agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilly, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewis, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanhon Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James B. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,629; Allen R. Jensen, Reg. No. 28,224; Lori Ann Johnson, Reg. No. 34,498; David A. Manspeizer, Reg. No. 37,540; Leslie A. McDonell, Reg. No. 34,872; Matthew T. Latimer, Reg. No. 44,024; Charles F. Costello, Reg. No. 27,324;

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Barbara V. Maurer, Reg. No. 31,278; Karen M. Dellerman, Reg. No. 33,692; David T. Banchik, Reg. No. 36,439; Fernando A. Borrego, Reg. No. 34,780; Mary E. Golota, Reg. No. 36,814; Anne G. Sabourin, Reg. No. 33,772; Brian W. Stegman, Reg. No. 30,977; Gerald L. Coon, Reg. No. 29,910; Mark A. Westhafer, Reg. No. 42,220; Elaine T. Sale, Reg. No. 41,286; Mary K. Cameron, Reg. No. 34,789; and Michael F. Morgan, Reg. No. 42,906.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Post Office Address: same as above		
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Post Office Address: same as above		
Full Name of Third Inventor: Sanford SILVERMAN	Inventor's Signature:	Date:
Residence: 10 N. Valley Rd., Roosevelt, New Jersey 08555		Citizenship: U.S.A.
Post Office Address: P.O. Box 657, Roosevelt, New Jersey 08555		

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<110> PAUSCH, MARK H.
LAI, MARGARET
SILVERMAN, SANFORD
BIRSAN, CAMELIA
BAUMBAUCH, WILLIAM
TSENG, EUGENE
KAJKOWSKI, EILEEN M.
OZENBERGER, BRADLEY A.

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 Oligonucleotide

<400> 36
 atccagatct aaaaatgtac ggaaaccaga cgaacgg

37

<210> 37
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<400> 37

taagctcgag ttatctaatt gtagacgagg cg

32

<210> 38

<211> 30

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 38

aaagatcca ggaactgtat aattaaagta

30

<210> 39

<211> 27

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 39

atgtctagaa attaacaaca ataaaga

27

<210> 40

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 40

atttctagac attgtttcat taattga

27

<210> 41

<211> 30

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 41

tttgtcgact tatctcatca ctggcattta

30

<210> 42

<211> 31

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 42

aagtggatcc attgtttcga aggaattaca g

31

<210> 43

<211> 52

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 43

agctggatcc tcaaaacaaa ccacaattctt taagggttttg ctggatgatt ag

52

<210> 44

<211> 52

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<400> 44

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52

<210> 45

<211> 52

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 45

agctggatcc tcaatacaaaa ccacaacctt taagggttttg ctggatgatt ag

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<210> 46

<211> 52

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
Oligonucleotide

<400> 46

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52

<210> 47

<211> 52

<212> DNA
 <213> Artificial Sequence

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<400> 47

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<210> 48

<211> 52

<212> DNA

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<400> 48

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<210> 49

<211> 52

<212> DNA

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<400> 49

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<210> 50

<211> 52

<212> DNA

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<210> 51

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<400> 51

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<210> 52
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<223> Description of Artificial Sequence:
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<400> 52

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52

<210> 53
 <211> 5
 <212> PRT
 <213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 53

Lys Ile Gly Ile Ile
 1 5

<210> 54
 <211> 5
 <212> PRT
 <213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 54

Asp Cys Gly Leu Phe
 1 5

<210> 55
 <211> 5
 <212> PRT
 <213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 55

Glu Cys Gly Leu Phe
 1 5

<210> 56
 <211> 5
 <212> PRT
 <213> Unknown

<220>
 <223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 56
 Gly Cys Gly Leu Tyr
 1 5

<210> 57
 <211> 5
 <212> PRT
 <213> Unknown

<220>
 <223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 57
 Tyr Ile Gly Leu Cys
 1 5

<210> 58
 <211> 4
 <212> PRT
 <213> Unknown

<220>
 <223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 58
 Glu Tyr Asn Leu
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<210> 59
 <211> 5
 <212> PRT
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<220>
 <223> Description of Unknown Organism: Mammalian G alpha
 protein carboxy-terminus

<400> 59
 Asp Ile Met Leu Gln
 1 5

<210> 60
 <211> 5
 <212> PRT
 <213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
protein carboxy-terminus

<400> 60

Gln Leu Met Leu Glu
1 5

<210> 61

<211> 5

<212> PRT

<213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
protein carboxy-terminus

<400> 61

Glu Asn Phe Leu Val
1 5

<210> 62

<211> 5

<212> PRT

<213> Unknown

<220>

<223> Description of Unknown Organism: Mammalian G alpha
protein carboxy-terminus

<400> 62

Glu Ile Asn Leu Leu
1 5

<210> 63

<211> 5

<212> PRT

<213> Unknown

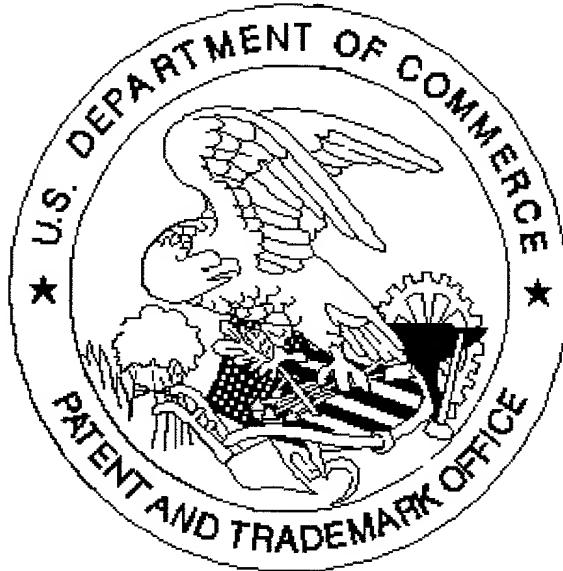
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<223> Description of Unknown Organism: Mammalian G alpha
protein carboxy-terminus

<400> 63

Gln Tyr Glu Leu Leu
1 5

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